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INTERFERENCE BETWEEN INFLUENZA AND VACCINIA VIRUSES.

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THERE are many examples in the literature of interference between influenza and unrelated viruses, *e.g.*, epidemic kerato-conjunctivitis (Henle and Henle, 1945), Eastern and Western equine encephalomyelitis (Henle and Henle, 1945; Vilches and Hirst, 1947), yellow fever (Lennette and Koprowski, 1946), mumps (Isaacs and Edney, 1950; Henle, 1950) and there would be little excuse for adding another to the list were it not that interference between influenza and vaccinia viruses shows novel features which may throw some light on the interference phenomenon. These are described in this and the following paper. Andrewes (1942) earlier found some evidence that influenza virus might inhibit the growth of vaccinia in chick embryo tissue cultures and the present results extend his findings.

MATERIALS AND METHODS.

Influenza virus : strain PR8.

The seed of this strain, kept at −70°, was passed in eggs by the allantoic route. The harvested fluid was used immediately or stored at −70° until use.

For some experiments PR8 virus was inactivated by exposing dialysed allantoic fluid to ultraviolet (U.V.) irradiation or by heating allantoic fluid at 56° for 30 min.

A/England/1/51 and B/Crawley influenza viruses.

These strains were used in the same conditions as PR8. In addition, some experiments were done with virus after purification by adsorption to fowl red cells and elution into normal saline.

Vaccinia virus.

Eggs incubated for 12 days were infected on the chorio-allantoic membrane with vaccinia virus seed. After incubation for 3 days at 35° the membranes covered with confluent pocks were harvested, ground in mortars with glass powder, suspended in broth saline, 1 ml. for each membrane, spun lightly and the supernatants kept at −70° as seed.

Fowl cells.

Fowl red cells of two types were used, vaccinia-positive or -negative, according to whether they are agglutinated or not by vaccinia virus (Stone, 1946). The cells were washed three times and made up in saline in 0·5 per cent suspension (v/v).

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Broth saline.

Normal saline containing 50 per cent of Hedley Wright broth.

Influenza virus haemagglutination.

Serial twofold dilutions of infected allantoic fluid were made in saline and to each tube was added an equal volume (0·25 ml.) of 0·5 per cent chicken red cell suspension. The mixtures were left at room temperature and the results read by a pattern test. Agglutination titres describe the number of agglutinating units (A.D.) per 0·25 ml. of undiluted allantoic fluid and are expressed as the reciprocal of the initial dilution of virus at the partial agglutination end-point.

Titration of haemagglutinin for vaccinia-positive cells.

After centrifugation of the ground chorio-allantoic membranes infected with vaccinia virus, the supernatant was removed and titrated with vaccinia-positive chicken red cells. Vaccinia-negative red cells were used to exclude the presence of influenzal haemagglutinins.

Interference experiments.

For interference experiments the chorio-allantoic membranes of fertile 12-day eggs were dropped (Beveridge and Burnet, 1946). Control eggs received 0·5 ml. of broth saline on the chorio-allantoic membrane. In the interference groups, each egg received 0·5 ml. of broth saline containing an appropriate amount of influenza virus. The eggs were incubated at 35° for 24 hr., then challenged by inoculation on the chorion with 0·1 ml. of vaccinia virus seed in broth saline at a dilution which gave the greatest number of readily countable pocks in the control membranes. With one sample of vaccinia virus seed, we used a $10^{-5.3}$ dilution, with another $10^{-4.5}$.

The eggs were incubated at 35° for 3 days after challenge. Thereafter, the "dropped" part of the membrane of each egg was harvested and the pocks counted. The membranes from the same group were then pooled, ground in a mortar with glass powder and suspended in 1 ml. of broth saline for each membrane. After spinning, the supernatant was tested for its capacity to agglutinate vaccinia-positive red cells.

RESULTS.

Interference between inactive influenza and vaccinia viruses in ovo.

Preliminary experiments were done with PR8 irradiated with U.V. and vaccinia viruses. Table I shows typical results.

TABLE I.—*Interference by U.V.-irradiated Influenza with the Multiplication of Vaccinia Virus.*

	Amount of U.V.-treated PR8 used :			Amount of heated Crawley used :	
	500 A.D.	50 A.D.	0.	1000 A.D.	0.
Number of pocks* . .	14	69	60	6	29
Vaccinial agglutinin titre† .	16	384	384	<1	48

U.V.-treated PR8 was a good interfering agent as shown by pock counting or by vaccinial haemagglutination titration; the haemagglutination titration was, however, a more sensitive measure of interference than the number of pocks, but it was also observed that the pocks in the test eggs tended to be smaller than in the controls. The smallest dose of U.V.-treated PR8 which regularly produced significant interference was about 500 A.D. In these experiments the PR8 was injected 24 hr. before the vaccinia virus. When the two viruses

* In this and the following Tables the number of pocks is expressed as the arithmetic mean of a group of 6 eggs.

† In this and the following Tables the vaccinial agglutinin titre is the haemagglutinin titre 0·25 ml. of a suspension of 6 membranes in 6 ml. of broth-saline.

were inoculated with an interval of only 1 hr., or together, or if the vaccinia preceded the PR8, no interference was found (see Table II).

TABLE II.—*Effect of Varying the Time Interval between Influenza and Vaccinia Virus Inoculations on the Degree of Interference.*

	Inoculation of influenza virus:			
	24 hr. before vaccinia.	With vaccinia.	24 hr. after vaccinia.	*Nil* (control).
Number of pocks . . .	5	32	>100	57
Vaccinial agglutinin titre . .	4	128	>500	256

Active influenza virus or virus heated at 56° for 30 min. was also a good interfering agent. An experiment with heated Crawley is illustrated in Table I.

Our preparations of active or inactive virus appeared to have about the same interfering potency, *i.e.*, interference was generally induced by about 100–500 A.D. of virus in different preparations.

Interference between purified influenza virus and vaccinia virus.

Influenza virus A/England/1/51 purified by adsorption on fowl red cells followed by elution into saline was tested as an interfering agent in the same experimental conditions as the infected allantoic fluid. The purified virus was an active interfering agent while the absorbed allantoic fluid had lost its interfering activity (Table III).

TABLE III.—*Interference with Vaccinia by a Preparation of Influenza Virus, after Adsorption to Red Cells and Elution.*

	Interfering virus preparation (A/ENG/1/51).					
	Untreated Virus.		Absorbed with red cells.	Eluate.		Control.
	1000 A.D.	100 A.D.	< 10 A.D.	1000 A.D.	100 A.D.	0
Number of pocks . .	2	6	46	1	6	50
Vaccinial agglutinin titre .	<1	3	128	<1	2	128

Lack of interference by vaccinia with influenza viruses.

Experiments were done to see if vaccinia virus would inhibit the development of influenza virus in the allantoic cavity of fertile eggs.

Three groups of 10-day-old eggs were inoculated allantoically with 0·05 ml. of broth saline in the first group and 0·05 ml. of undiluted vaccinia virus seed in the second and third groups.

After incubation at 35° for 24 hr. the eggs of the first and second groups were challenged with 10^4–10^5 ID_{50} of PR8 influenza virus, the third group received 0·05 ml. of broth saline. After a further 48 hr. at 35° the allantoic fluids were harvested separately and tested for their agglutination titre with vaccinia-positive and negative fowl red cells. It was found that vaccinia did not modify the growth of PR8 virus.

Experiments in mice infected intranasally showed no interference by undiluted vaccinia virus with the lung lesions produced by influenza virus. Our strain of vaccinia virus produced insignificant lesions in the lungs of mice.

Interference between influenza and vaccinia virus in rabbits.

Rabbits of 1500 g. wt. were used for these experiments. The skin of the flanks was clipped and the animals were inoculated intradermally with 0·05 ml. broth saline containing 500 A.D. of influenza virus or 0·05 ml. of broth saline; generally 3–4 experimental and 3–4 control areas were used in each rabbit. One day later they were again injected in the same areas with 0·05 ml. of a 10^{-5} dilution of vaccinia virus in broth saline. The evolution of vaccinial lesions was watched during the next 1–2 weeks, and it was found that both live and inactive (U.V.) PR8 inhibited significantly the development of vaccinial lesions. In a representative experiment there were 8 large and 4 moderate lesions in control sites compared with 2 slight lesions and 14 sites showing no lesions in areas previously injected with 500 A.D. of PR8 virus.

DISCUSSION AND SUMMARY.

Active or inactivated influenza virus A or B in 100–500 agglutinating doses inhibited the development of vaccinia virus lesions on the chick chorion and in the rabbit skin. The inhibition of vaccinial growth on the chick chorion could be measured by the reduction in the number of pocks, or more easily, by the reduction in the titre of vaccinial haemagglutinin in the membrane. The interference process in the chorion required some time to be established, as was observed in the interference between heated and active influenza viruses in the allantoic cavity (Fazekas de St. Groth, Isaacs and Edney 1952). No evidence of interference by vaccinia with influenza virus has been found in eggs or mice. The identity of the interfering agent with the influenza virus rests on the demonstration that both are absorbed from allantoic fluid by red cells and can be eluted quantitatively from the cells, and on the evidence in the accompanying paper that the interfering action is neutralised by influenza virus antiserum.

The present experiments were commenced because it had been found earlier (Isaacs and Fulton, 1953) that inactivated influenza virus preparations similar to those used in the present work failed to inhibit the production of "soluble" antigen which occurs when active influenza virus is inoculated on the chick chorion. Such preparations do inhibit the small rise of infectivity which normally occurs following the inoculation of about 100 ID_{50} of PR8 virus on the chorion, and they greatly inhibit the development of influenza virus in the chick allantois. These results suggest that influenza and vaccinia viruses require similar factors for their development and the possible nature of one of these factors is discussed in the following paper.

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EFFECT OF INHIBITORS ON INTERFERENCE BETWEEN INFLUENZA AND VACCINIA VIRUSES.

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THE demonstration that influenza virus interfered with the growth of vaccinia on the chick chorion (Depoux and Isaacs, 1954) suggested that it might be of interest to investigate the effect of known inhibitors on both these viruses and on the interference between the two. This paper describes an investigation of the effect of the receptor-destroying enzyme (RDE) of *V. cholerae* (Burnet and Stone, 1947) on the growth of influenza and vaccinia viruses on the chick chorion and on the interference between these viruses. The behaviour of RDE is contrasted with that of influenza virus antiserum and of the normal serum inhibitors of influenza virus agglutination.

MATERIALS AND METHODS.

In addition to the materials and methods described in the preceding paper, the following were used.

Sera.—Normal serum was taken from ferrets before, and immune serum 12 days after intranasal inoculation of 1 ml. of a 10^{-2} dilution of infected allantoic fluid.

Receptor-destroying enzyme.—A purified preparation of RDE from *V. cholerae* was made available through the courtesy of Sir Macfarlane Burnet. It was diluted in acetate buffer pH 6·2 containing 0·1 per cent $CaCl_2$ and titrated by the method of Burnet and Stone (1947).

Titration of "soluble" antigen.—To the "dropped" portion of chorio-allantoic membrane 1 ml. of saline was added and this was frozen rapidly to −70° and thawed slowly at room temperature three times. After light centrifugation, the supernatant fluid was used as antigen. It was titrated in 0·1 ml. volumes with 2·5 M.H.D. of complement and 3 minimal fixing doses of human convalescent influenza A serum. In these conditions the content of antibody to the soluble antigen only is measured. The titres in the Tables show the initial dilution of antigen at the 50 per cent fixation end-point, expressed as the number of units fixed per membrane.

RESULTS.

Effect of RDE on interference by influenza with vaccinia virus.

In the type of interference experiment previously described (Depoux and Isaacs, 1954) about 200–500 agglutinating doses (A.D.) of influenza virus were inoculated on the "dropped" portion of the chorio-allantoic membrane, and followed 24 hr. later by an inoculum of vaccinia virus which produced an easily countable number of pocks (about 50).

Interference was assessed by a reduction in the number of pocks and in the amount of vaccinial haemagglutinin compared with those occurring in control eggs. The effect of RDE on this reaction was tested by inoculating 1000 units

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of RDE before the interfering virus. Table I shows the results of an experiment in which eggs were inoculated with influenza virus alone, RDE alone, RDE followed by influenza virus and broth-saline as a control. After 24 hr. all eggs were challenged with vaccinia virus and the results assessed after incubation for 3 more days at 35°.

TABLE I.—*Effect of Influenza Virus and RDE on the Growth of Vaccinia.*

1st inoculum	*Nil*	RDE	RDE	Broth-saline
2nd inoculum	Influenza virus	*Nil*	Influenza virus	*Nil*
Mean number of pocks	2	34	31	43
Mean vaccinial agglutinin titre	2	640	160	1280

3rd inoculum—all eggs inoculated with 0·1 ml. of vaccinia dilution.
Influenza virus—1000 A.D. of Influenza A virus, Melbourne strain.
RDE—1000 units of purified receptor-destroying enzyme in calcium acetate buffer pH 6·2.
2nd inoculum was given 4 hr. after the first and was followed by vaccinia virus 24 hr. later.
6 eggs were used in each group.

Table I shows that the influenza virus alone produced considerable inhibition of vaccinial growth as shown both by pock counts and by development of vaccinial haemagglutinin. RDE alone had no significant action on vaccinial growth but it inhibited the influenzal interfering action. In this experiment the RDE injection preceded the influenza virus by 4 hr. However it was interesting to note that when the RDE was given 2 hr. after the influenza virus, significant inhibition of the influenzal interfering action again occurred. An experiment illustrating this is shown in Table II.

TABLE II.—*Effect of RDE Given 2 Hours after Influenza Virus on the Growth of Vaccinia.*

1st inoculum	Influenza virus	Influenza virus	Broth-saline
2nd inoculum	*Nil*	RDE	*Nil*
Mean number of pocks	5	24	60
Mean vaccinial agglutinin titre	1	96	480

3rd inoculum—all eggs inoculated with 0·1 ml. of vaccinia dilution.
Influenza virus—200 A.D. of A/ENG/1/51. RDE—1000 units.
2nd inoculum was given 2 hr. after the first.
6 eggs per group.

Effect of RDE on growth of influenza virus in the chick chorion.

Influenza virus grown in the chick chorion has been shown to undergo a single cycle of multiplication (Fulton and Isaacs, 1953). Development of the virus is readily followed by the appearance in the membrane of " soluble " complement-fixing antigen and there is a linear relation between the log-dosage of virus inoculated and the log-amount of " soluble " antigen produced. The effect of RDE on this cycle was investigated by following the production of " soluble " complement-fixing antigen.

RDE, 1000 units, made up in calcium acetate buffer was inoculated on the chorion, followed 1 hr. later by 200 A.D. of influenza virus (A/England/1/51 strain). Controls received calcium acetate and influenza virus. One day later

the membranes were harvested and tested for their " soluble " antigen content. No " soluble " antigen, or only traces of antigen, could be detected in the eggs inoculated with RDE. Controls showed that RDE in the same dosage had no effect on the *in vitro* combination of antigen and antibody in the complement fixation test.

In further experiments it was found that RDE given 2 hr. after the virus caused a 4-fold or greater reduction in the " soluble " antigen titre, compared with controls given calcium acetate buffer. An experiment illustrating this is shown in Table III.

TABLE III.—*Effect of RDE Given 2 Hours after Influenza Virus on the Production of Influenzal " Soluble " Antigen in the Chick Chorion.*

1st inoculum	Influenza virus	Influenza virus	Influenza virus
2nd inoculum	*Nil*	Calcium acetate buffer.	1000 units RDE
Titres of " soluble " antigen in individual chorio-allantoic membranes	60	30	<10
	240	15	10
	100	30	<10
	120	80	<10
	15	30	<10
	120	30	<10

2nd inoculum given 2 hr. after the 1st.
Influenza virus—200 A.D. of A/ENG/1/51 virus.

In this experiment it appeared that calcium acetate alone may have reduced slightly the yield of soluble antigen but RDE had a much greater effect.

Effect of RDE on adsorption of influenza virus to the chorion.

The first and most obvious explanation of these results is that RDE prevented the adsorption of the influenza virus to the chick chorionic cells. RDE is known to prevent the adsorption of influenza virus to the surface of chick red cells (Burnet, McCrea and Stone, 1946), the chick allantoic membrane (Stone, 1948) and the respiratory epithelium of the mouse lung (Fazekas de St. Groth, 1948). On the other hand, it was surprising to find that RDE still reduced significantly the yield of influenza " soluble " antigen and inhibited the interfering action when it was inoculated 2 hr. after the influenza virus, at a time when one might have expected that most of the virus would have been absorbed.

A direct test of the effect of RDE on the absorption of influenza virus was therefore made. Two hundred A.D. of A/England/1/51 virus were inoculated on the chorion of 12 eggs. Half the eggs were inoculated 2 hr. later with 1000 units of RDE and the remainder with an equal volume of calcium acetate buffer. After 1 hr. incubation the fluids were harvested and tested for their agglutinin titres ; dilutions of virus were made in 2 per cent citrate saline and the red cells allowed to settle at 2°. Controls of virus plus buffer or RDE were incubated at 35° along with the eggs. The results showed that in the eggs receiving calcium acetate, over 95 per cent of the influenza virus could no longer be detected 3 hr. after its inoculation on the chorion. In eggs treated with RDE slightly more virus was recovered, but nevertheless 87·5 per cent of the inoculated virus could not be recovered and was presumably adsorbed to and taken up by the chorionic cells. In another and similar experiment about 90 per cent of virus was adsorbed

in eggs inoculated with calcium acetate and 80 per cent in eggs given RDE; the same amount of adsorption was found when RDE was given 1 hr. before or 2 hr. after the influenza virus.

These experiments suggest, therefore, that when 200 A.D. of influenza virus were inoculated on the chick chorion the greater part of the virus was adsorbed to the chorionic cells within 3 hr. at 35°. An additional small amount of the inoculum was not adsorbed when 1000 units of RDE were given 1 hr. before or 2 hr. after the virus, but nevertheless the greater part of the virus (about 80 per cent) was taken up by the cells and could not be released by the action of RDE. Indirect confirmation of this conclusion was obtained by studying the action of immune and normal serum on the growth of influenza virus on the chorion and on its effect on the interference between influenza and vaccinia viruses.

Effect of immune and normal ferret serum on interference by influenza with vaccinia virus.

When influenza virus antiserum diluted 1/10 was mixed with the homologous virus the interference with vaccinia virus growth was readily abolished. The controls for this experiment consisted of influenza virus mixed with normal ferret serum (heated at 56° for 30 min.) and it was surprising to find that in this case, too, some inhibition of interference occurred. The action of normal ferret serum was traced to its content of normal serum inhibitor of influenza virus agglutination and could be abolished by pre-treating the serum with trypsin or with RDE. Table IV shows the results of a characteristic experiment illustrating these points.

TABLE IV.—*Effect of Trypsin or RDE-treated Immune and Normal Ferret Sera Mixed with Influenza Virus on Interference with Vaccinia Virus Growth.*

		1st inoculum: 200 A.D. of A/ENG/1/51 virus mixed with—						
	Broth-saline.	*Nil.*	Heated NFS.	Trypsin-treated NFS.	RDE-treated NFS.	Heated IFS.	Trypsin-treated IFS.	RDE-treated IFS.
Mean number of pocks	58	10	40	10	17	51	66	42
Mean vaccinial agglutinin titre	120	1·5	40	1·5	8	120	240	80

2nd inoculum—all eggs inoculated with 0·1 ml. of vaccinia dilution.
NFS—Normal ferret serum.
IFS—Immune ferret serum.
Table shows mean number of pocks and vaccinial agglutinin titres of groups of 6 eggs.

It will be seen that the interference with vaccinia by influenza virus was prevented by mixing the influenza virus with immune serum; serum heated at 56° or pre-treated with trypsin or RDE and then heated at 56° was equally effective. Normal ferret serum heated at 56° mixed with influenza virus reduced greatly its interfering action but in this case pre-treating the serum with trypsin, and to a lesser extent RDE, abolished the action of the serum. We conclude therefore that the interfering action of influenza virus can be prevented by mixing the virus with specific antibody or with normal serum inhibitor.

The effect of immune and normal ferret serum given 2 hr. after the influenza virus was next investigated. The results are shown in Table V.

TABLE V.—*Effect of Immune and Normal Ferret Serum Given With or 2 Hours after Influenza Virus on the Growth of Vaccinia.*

1st inoculum	Broth-saline	Influenza virus	Influenza virus + IFS	Influenza virus + NFS	Influenza virus	Influenza virus
2nd inoculum	*Nil*	*Nil*	*Nil*	*Nil*	IFS	NFS
Mean number of pocks	60	2	51	43	15	7
Mean vaccinial agglutinin titre	160	<1	160	80	4	2

3rd inoculum—all eggs inoculated with 0·1 ml. of vaccinia dilution.
Influenza virus—500 A.D. of A/ENG/1/51.
2nd inoculum given 2 hr. after the 1st.
IFS—Immune ferret serum.
NFS—Normal ferret serum.

The Table shows that the interference with vaccinia by influenza virus is readily inhibited when immune or normal ferret serum is inoculated mixed with the influenza virus. The same amount of immune or normal ferret serum given 2 hr. after the influenza virus did not significantly inhibit the interference. The slight inhibition observed may have been due to an action on the small amount of influenza virus still unabsorbed 2 hr. after inoculation.

Effect of immune and normal ferret serum on "soluble" antigen production in the chick chorion.

When influenza virus was mixed with normal or immune ferret serum its ability to produce "soluble" antigen after inoculation on the chick chorion was greatly inhibited. However, the same amount of serum given 2 hr. after the virus had no effect on the subsequent production of "soluble" antigen. An experiment illustrating this is shown in Table VI.

TABLE VI.—*Effect of Immune and Normal Ferret Serum Given With or 2 Hours after Influenza Virus on the Development of "Soluble" Antigen in the Chick Chorion.*

1st inoculum	Influenza virus	Influenza virus + NFS	Influenza virus	Influenza virus + IFS	Influenza virus
2nd inoculum	*Nil*	*Nil*	NFS	*Nil*	IFS
Titres of "soluble" antigen in individual chorio-allantoic membranes	60	<10	60	<10	120
	80	<10	30	<10	60
	80	<10	60	<10	30
	60	<10	15	<10	60
	15	<10	120	<10	20
	60	<10	40	<10	—

NFS—Normal ferret serum.
IFS—Immune ferret serum.

We conclude from the results of experiments of this type and of that shown in Table V that 2 hr. after inoculation on the chorion the bulk of the influenza virus inoculum can no longer be neutralised by specific antibody or normal serum inhibitor.

DISCUSSION.

These experiments show that the receptor-destroying enzyme of *V. cholerae* inhibits two actions of influenza virus in the chick chorion, its ability to produce " soluble " complement-fixing antigen and to interfere with the growth of vaccinia virus. Both effects were observed if RDE was inoculated before, or 2 hours after the influenza virus. The first explanation which came to mind was that RDE inhibited adsorption of influenza virus to the chick chorionic cells but two observations showed that this factor did not play a significant rôle in these results. First, it was found that 2–3 hours after inoculating 200 A.D. of influenza virus on the chick chorion the great majority of the inoculated virus could no longer be recovered. The injection of RDE before or 2 hours after the virus increased only very slightly the recovery of virus. The dosages of RDE and virus are probably all-important in this connection. Thus, an amount of RDE sufficient to prevent agglutination of chick red cells by a small dose of influenza virus does not prevent agglutination by a larger dose (Stone, 1947 ; Hirst, 1950). Similarly, an amount of RDE (3200 units) sufficient to prevent the initiation of infection by small doses of influenza virus in the allantoic cavity of the chick embryo does not prevent infection by larger doses of the same virus (Stone, 1948). Also, Stone (1948) has shown that 3200 units of RDE inhibited the adsorption of 10–30 A.D. of virus in the allantoic cavity, but 640 units of RDE had no effect. Secondly specific antibody and normal ferret serum inhibitors reversed both actions of influenza virus when the serum and virus were mixed, but not when the serum was given 2 hours after the virus. This finding supports the idea that 2 hours after inoculation most of the influenza virus is taken up by the cells and is no longer accessible to the action of serum. Henle and Henle (1949) noted that immune serum inoculated into the allantoic cavity 30 minutes after a large dose of influenza virus had no significant effect on the subsequent yield of virus.

We are forced to conclude therefore, that RDE acts when the influenza virus is intracellular. Presumably, some RDE is capable of entering chorionic cells ; this appears to be the simplest explanation of its activity although there is no direct experimental evidence to support it. On the other hand, both virus and RDE are adsorbed to these cells and if the cells are capable of taking up virus it is difficult to see why they should be unable to take up the much smaller RDE molecule. The substrate for this enzyme is the mucoprotein chorio-allantoic membrane inhibitor which is probably present in chorionic as well as allantoic cells, although the fact that the bulk of membrane inhibitor is inactivated following growth of influenza virus in the allantoic cells (Edney and Isaacs, 1950 ; Liu and Henle, 1951) suggests that there is less inhibitor in the chorionic than in the allantoic cells. It would appear, therefore, that some interaction between influenza virus and chorio-allantoic membrane inhibitor is a necessary step in the formation of " soluble " antigen and in the interference with vaccinia virus in the chick chorion, and that RDE interrupts this step. It would be hazardous to speculate further in the present state of knowledge.

There is an intriguing relationship between the growth requirements of influenza and vaccinia viruses in the chick chorion. The production of " soluble " antigen by influenza virus is inhibited by RDE but not by inactivated influenza virus (Isaacs and Fulton, 1953). On the other hand, the growth of vaccinia virus is inhibited by inactivated influenza virus but not by RDE.

SUMMARY.

RDE (1000 units) given before, or 2 hours after chorionic inoculation of influenza virus inhibited two of the activities of the virus, its ability to interfere with the growth of vaccinia and to produce " soluble " antigen. Influenza virus antibody and normal ferret serum inhibitor inhibited these activities when they were inoculated with, but not 2 hours after the virus. RDE did not prevent adsorption of the greater part of the influenza virus to the chorionic cells and its site of action is thought to be intracellular.

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A SEROLOGICAL INVESTIGATION OF SIX ENCEPHALITIS VIRUSES ISOLATED IN MALAYA.

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SEVERAL virus isolations have been made from the brains of fatal human encephalitis cases in Malaya. Clinically all these cases were compatible with Japanese B encephalitis virus infection, and histological examination of the brains confirmed this. Careful examination of these viruses however revealed that they were not all identical and the findings with six strains are reported in this paper.

MATERIALS AND METHODS.

Virus strains.

These were designated: Muar, Tengah, G.H.1/52, G.H.3/52, M.7/52 and M.1/53. The Nakayama strain of Japanese type B encephalitis virus was used for comparison. Viruses were kept in ampoules as a 20 per cent infected mouse brain suspension in 50 per cent inactivated rabbit serum saline and stored in alcohol-dry ice mixture.

Animal inoculation.

Eggs.—For primary isolation selected pieces of brain from fatal cases were ground up in serum saline containing 1000 units penicillin and 1 mg. streptomycin per ml. to give a 20 per cent suspension. Of this suspension 0·05 ml. was inoculated on to the chorio-allantoic membrane of 10-day fowl eggs. After 3 days' incubation at 37° the eggs were opened and the membranes examined. The membranes usually exhibited rather indefinite pock-like lesions and passage of these membranes as a 1 per cent suspension to further eggs resulted in the appearance of definite pocks.

Mice.—Three week old Swiss white mice were inoculated with 0·03 ml. intracerebrally or 0·2 ml. by the intraperitoneal route. For primary isolations 20 per cent brain suspension in 10 per cent rabbit serum saline was used.

Guinea-pigs.—Young guinea-pigs received 0·2 ml. intracerebrally or 0·5 ml. intraperitoneally of 10 per cent infected mouse brain suspension in 10 per cent rabbit serum saline.

Rabbits.—Of the 10 per cent infected mouse brain suspension in 10 per cent rabbit serum saline 0·3 ml. was inoculated intracerebrally or 0·5 ml. intraperitoneally.

Monkeys.—When monkeys were used for testing the pathogenicity of the virus strains they received 0·3 ml. intracerebrally of a 10 per cent infected mouse brain suspension in 10 per cent rabbit serum saline.

Vaccines.

Three week old mice were inoculated intracerebrally with a 1 in 100 dilution of infected mouse brain suspension. When the animals were prostrate they were killed, their brains removed and emulsified in 10 per cent rabbit serum saline to give a 10 per cent suspension. Commercial formaldehyde was added to 0·5 per cent and the mixture kept in the refrigerator until it was no longer virulent to mice on intracerebral inoculation, usually 14 days. This vaccine was used for the production of hyperimmune sera and for preliminary inoculation in cross resistance tests.

Hyperimmune sera.

Mice.—Mice received the following inoculations: 1st and 3rd day, 0·3 ml. of 10 per cent formolised vaccine intraperitoneally and on the 8th, 13th, 18th and 23rd day—0·3, 0·5, 0·5, and 0·75 ml. respectively of a dilution of living virus suspension intraperitoneally. Five days after the last inoculation they were bled from the heart under ether anaesthesia, the blood pooled and allowed to clot. Separated serum was stored at − 20°.

Rabbits.—Rabbits were given 1 ml. doses of vaccine intraperitoneally at intervals of 2 days followed by 3 injections intraperitoneally of 1 ml. of 10 per cent live virus suspension, afterwards 2 injections of 2 ml. and finally 6 injections of 4 ml., all at 5-day intervals. They were bled from the ear vein and the serum stored at − 20°.

Antigen for complement fixation.

Infected mouse brains, ground up in 2 per cent inactivated guinea-pig serum saline to give a 10 per cent suspension, were kept in the refrigerator overnight. After spinning in the horizontal centrifuge at 2500 r.p.m. for 30 min., the clear supernatant fluid was frozen with a mixture of alcohol and dry ice and later thawed. This procedure, repeated five times, produced flocculation, and spinning at 3000 r.p.m. in an angle centrifuge for 60 min. yielded a clear supernatant fluid. Merthiolate was added to give a final dilution of 1 in 10,000 and this preparation diluted 1 in 5 was used as antigen.

Cross neutralisation tests.

In the test, tubes containing 0·2 ml. of unheated, undiluted immune rabbit sera were mixed with 0·2 ml. of dilutions of 20 per cent suspensions of mouse brain infected with the virus under test. After incubation of the tubes for 2 hr. at 37° in the water bath, mice were inoculated intracerebrally with the mixture. Six mice were used for each virus dilution and observed for 14 days. A normal rabbit serum known to contain no neutralising antibodies was used as a control in each test. The technique was essentially that described by Hammon (1948) in *Diagnostic Procedures for Virus and Rickettsial Diseases.*

The LD_{50} end-points were calculated by the method of Reed and Muench (1938) and the neutralising capacity of sera expressed by the neutralisation index (the difference in log dilution of LD_{50} end-points of virus-serum mixtures between the serum in question and the normal rabbit serum control). Two antisera were tested on each occasion against homologous and heterologous virus.

Cross complement fixation tests.

These followed the technique outlined by Casals and Palacios (1941) with overnight incubation of mixtures of immune mouse serum, antigen and complement at 5°. Complement titrations were also carried out with overnight incubation at 5° in the presence of antigen. Antigens from the six virus strains under test were prepared from mouse brains infected with these strains and antigen prepared from normal mouse brain by the same procedure used as a control. Immune sera were tested in pairs as in the cross neutralisation test.

Cross resistance tests.

Three week old Swiss white mice were immunised by the same procedure as described for the preparation of mouse antisera and challenged intracerebrally with 0·03 ml. of virus dilutions of infected mouse brain suspensions. The difference in LD_{50} of the challenging virus between protected and unprotected mice of the same age gave the number of intracerebral LD_{50} doses of virus against which this immunisation protected. As in other tests two virus strains were compared together so that the results expressed the degree of protection of both against homologous and heterologous virus.

RESULTS.

Isolation.

The isolation of all the strains with the exception of M.7/52 was accomplished both by intracerebral inoculation of mice and chorio-allantoic inoculation of

developing eggs. Strain M.7/52 was isolated in eggs but despite repeated attempts, isolation proved impossible in mice. After several chorio-allantoic passages of this strain it was possible to produce encephalitic symptoms in 3-day old mice following intracerebral inoculation of ground-up membrane suspensions, and after several passages in young mice the virus infected 3-week old mice. A similar finding has been reported by French (1952) in the case of the Murray Valley encephalitis virus.

Pathogenicity of strains.

Infected mouse brains in 10 per cent suspensions were inoculated intracerebrally and intraperitoneally into mice, guinea-pigs, rabbits and monkeys. Table I shows the results.

The intracerebral inoculation of monkeys resulted in ataxia, paralysis, progressive weakness and finally death, with the exception of strains M.7/52 and M.1/53 which caused paralysis of one or more limbs but were never fatal. Muar differed from the other strains in that intracerebral inoculation of guinea-pigs always caused the death of some of the animals with encephalitic symptoms; usually one or two out of a batch of six died, but this was never found with other strains.

TABLE I.—*Pathogenicity of the Strains to Laboratory Animals.*

Virus strain.	Mice.		Guinea-pigs.		Rabbits.		Monkeys.
	IC.	IP.	IC.	IP.	IC.	IP.	IC.
Muar	++++	++	±	0	0	0	+++
Tengah	++++	++	0	0	0	0	+++
G.H.1/52	++++	++	0	0	0	0	+++
G.H.3/52	++++	++	0	0	0	0	+++
M.7/52	++++*	++*	0	0	0	0	+
M.1/53	++++	++	0	0	0	0	+
Nakayama	++++	++	0	0	0	0	+++

IC = Intracerebral inoculation.
IP = Intraperitoneal inoculation.
\+ = Degree of pathogenicity.
* = After the adaptation described.

Serological Analysis.

Cross neutralisation tests.

The neutralising capacity of antisera prepared in rabbits against the classical Nakayama strain and the virus under test was estimated both for homologous and heterologous strains. The results are given in Table II. Each strain is shown against the Nakayama strain in this way, because although the experiments were repeated several times, each experiment was conducted separately and Nakayama antiserum was often obtained from different immune rabbits and by different bleedings.

Allowing for experimental error, strains G.H.1/52, G.H.3/52, M.7/52 and M.1/53 are neutralised by Nakayama antisera to the same extent as the Nakayama virus; conversely antisera prepared against these strains have an equal neutralising capacity both against the homologous strain and the Nakayama virus.

The other two strains, Muar and Tengah, are neutralised by Nakayama antisera but the neutralising index of the serum is not so high as when tested

TABLE II.—*Cross Neutralisation: Comparison of Strains with Nakayama.*

Antiserum.	Virus. Nakayama.	G.H.1/52
Nakayama	5·52	5·05
G.H.1/52	5·25	5·53

Antiserum.	Virus. Nakayama.	G.H.3/52.
Nakayama	4·88	4·10
G.H.3/52	5·25	4·70

Antiserum.	Virus. Nakayama.	M.7/52.
Nakayama	5·0	5·25
M.7/52	4·84	4·77

Antiserum.	Virus. Nakayama.	M.1/53.
Nakayama	5·55	5·37
M.1/53	4·75	4·62

Antiserum.	Virus. Nakayama.	Muar.
Nakayama	5·12	4·50
Muar	3·09	5·20

Antiserum.	Virus. Nakayama.	Tengah.
Nakayama	5·65	4·60
Tengah	3·40	4·87

Figures = the log of the neutralisation index of the sera.

against the homologous virus. This difference is more marked when the neutralising capacity of Muar and Tengah antisera is estimated for homologous virus and the Nakayama virus, the same being true when Muar is tested against M.7/52 antisera. Muar and Tengah show complete identity in this test (Table III).

TABLE III.—*Cross Neutralisation.*

Antiserum.	Virus. Muar.	M.7/52.
Muar	4·34	3·80
M.7/52	3·09	4·95

Antiserum.	Virus. Muar.	Tengah.
Muar	4·84	5·10
Tengah	4·24	4·15

Figures = the log of the neutralisation index of the sera.

Although M.7/52 behaved like the Murray Valley encephalitis virus during isolation, it was not possible to demonstrate the difference in neutralisation with Japanese B encephalitis antisera reported by French (1952) in the case of the Murray Valley encephalitis virus.

This cross neutralisation test divides our viruses into two groups, one represented by the Nakayama, G.H.1/52, G.H.3/52, M.7/52 and M.1/53 strains and the other by Muar and Tengah.

Cross complement fixation tests.

The results are shown in Table IV set out in individual experiments as in Table II and for the same reason, that different samples of mouse antisera were used for each experiment. Strains Nakayama, G.H.1/52, G.H.3/52, M.7/52 and M.1/53 are practically identical by these tests, but the antigenic composition of Muar and Tengah differs from that of the members of this group.

TABLE IV.—*Cross Complement Fixation: Comparison of the Strains with Nakayama.*

Antiserum.	Antigen. Nakayama.	G.H.1/52.
Nakayama	128	128
G.H.1/52	128	128

Antiserum.	Antigen. Nakayama.	G.H.3/52.
Nakayama	32	32
G.H.3/52	32	64

Antiserum.	Antigen. Nayakama.	M.7/52.
Nakayama	128	256
M.7/52	128	256

Antiserum.	Antigen. Nakayama.	M.1/53.
Nakayama	16	16
M.1/53	32	32

Antiserum.	Antigen. Nakayama.	Muar.
Nakayama	16	4
Muar	8	64

Antiserum.	Antigen. Nakayama.	Tengah.
Nakayama	32	8
Tengah	8	32

Figures = the reciprocals of the titre.

Similar tests against the Muar strain given in Table V confirm the similarity of Muar and Tengah and emphasise the difference from strain M.7/52, selected as a representative of the major group.

TABLE V.—*Cross Complement Fixation.*

Antiserum.	Antigen. Muar.	Tengah.
Muar	32	32
Tengah	32	32

Antiserum.	Antigen. Muar.	M.7/52.
Muar	64	64
M.7/52	4	32

Figures = the reciprocals of the titre.

Cross resistance tests.

Although strains G.H.1/52, G.H.3/52, M.7/52 and M.1/53 behaved exactly like Nakayama virus in complement fixation and neutralisation tests, mice vaccinated with these strains received little or no protection against intracerebral challenge by the Nakayama strain. Mice vaccinated with the Nakayama strain however were protected against these strains (Table VI). This was a similar finding to that reported by Paterson *et al.* (1952) with the Kinrara strain isolated at Kuala Lumpur, Malaya.

Slight cross protection was found with the Muar and Nakayama strains, but none with Tengah and Nakayama.

Tests with Muar and Tengah shown in Table VII gave complete cross protection, but Muar gave no cross protection with M.7/52.

TABLE VI.—*Cross Resistance: Comparison of the Strains with Nakayama.*

Vaccination.	Virus. Nakayama.	G.H.1/52.
Nakayama	91	1510
G.H.1/52	0	340

Vaccination.	Virus. Nakayama.	G.H.3/52.
Nakayama	315	2400
G.H.3/52	0	280

Vaccination.	Virus. Nakayama.	M.7/52.
Nakayama	890	1780
M.7/52	0	1780

Vaccination.	Virus. Nakayama.	M.1/53.
Nakayama	440	6300
M.1/53	85	1175

Vaccination.	Virus. Nakayama.	Muar.
Nakayama	1700	100
Muar	110	2500

Vaccination.	Virus. Nakayama.	Tengah.
Nakayama	2140	0
Tengah	0	275

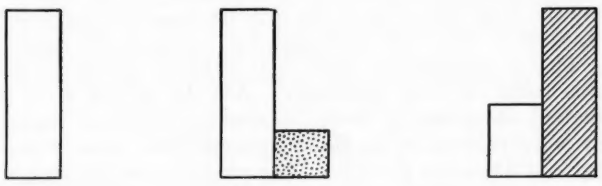
Figures = the number of intracerebral LD_{50} doses against which vaccination gave protection.

TABLE VII.—*Cross Resistance.*

Vaccination.	Virus. Muar.	Tengah.
Muar	2300	1050
Tengah	500	50

Vaccination.	Virus. Muar.	M.7/52.
Muar	235	0
M.7/52	0	2400

Figures = the number of intracerebral LD_{50} doses against which vaccination gave protection.



Type 1. G.H.1/52 G.H.3/52 M.7/52 M.1/53 . Type 2. Nakayama . Type 3. Muar Tengah

FIGURE.—Antigenic composition of the three serological types.

DISCUSSION.

These results show that the strains we have isolated, although they produce the same clinical symptoms and pathological lesions, are not homogeneous.

Failure to isolate M.7/52 in mice and the ease with which it was isolated in the chorio-allantoic membrane was suggestive of Murray Valley encephalitis virus

(French, 1952). The Murray Valley virus was however found to be non-pathogenic to monkeys but M.7/52 produced non-fatal paralysis. Our first attempts at infecting monkeys with M.7/52 and M.1/53 failed. Repeated failures led us to investigate the immunological state of monkeys and we found that a proportion of the local monkeys used possessed neutralising antibodies to these encephalitic viruses. Selection of monkeys with no neutralising antibodies to the strain whose pathogenicity we were testing, allowed us to elicit the paralysis produced by these two viruses. Presumably with the viruses of greater pathogenicity to monkeys any natural immune state was not sufficient to withstand the intracerebral challenge and therefore we had not previously encountered this problem. The failure to infect monkeys with Murray Valley virus could possibly be due to the same cause if preliminary serological examination of the animals had not been undertaken. Despite these points of similarity between M.7/52 and Murray Valley virus, we were unable to find differences in cross neutralisation with Nakayama antisera reported by French (1952) as occurring with the Murray Valley virus.

Neutralisation tests indicated that the Muar and Tengah strains were not identical with the other strains, Nakayama, G.H.1/52, G.H.3/52, M.7/52 and M.1/53. Antigens prepared from the Muar and Tengah strains gave some fixation with Nakayama antisera but not to so high a titre as that obtained with the homologous antigen. Similarly Nakayama antigen did not give the same degree of fixation with antisera to these two strains as did the homologous antigens. Cross resistance tests revealed little cross protection between these two strains and the Nakayama virus, but although they are not identical with the classical strain of Japanese Type B encephalitis virus, they have some antigenic relationship with it. This is demonstrated by the minor degree of cross reaction in complement fixation tests, their neutralisation to a certain extent by Nakayama antisera and the ability of antisera to these strains to give some neutralisation of the Nakayma virus. The two strains Muar and Tengah proved to be antigenically similar. Their possible identity with other encephalitis viruses related to Japanese B arose, notably the West Nile virus which is neutralised by Japanese B antiserum although its antiserum does not neutralise the Japanese B virus (Smithburn, 1942). The ability of antisera to Muar and Tengah viruses to neutralise the Nakayama virus ruled out this possibility. Are the strains Muar and Tengah sufficiently different antigenically from the classical strain of Japanese B virus to justify their being referred to as Malaya encephalitis virus or should they be regarded as types of Japanese B virus? The tendency on the part of workers in the field of neurotropic viruses seems to have been to designate strains according to the place of isolation, thus we have Murray Valley, St. Louis encephalitis, etc. To us this practice seems unjustified; the differences are often very small and the viruses have similar biological properties producing the same type of disease and lesions in susceptible animals. We prefer to regard the situation as with influenza viruses, a range of viruses of similar properties but divisible into different serological types.

Accepting this view, then, we have produced evidence of the existence of three serological types of Japanese B encephalitis virus—the classical Nakayama strain, a type represented by Muar and Tengah strains and a third type represented in this series by G.H.1/52, G.H.3/52, M.7/52 and M.1/53. This last type differs from the classical type in that vaccination of mice with these strains fails to protect

against intracerebral challenge with the classical strain, whereas vaccination of mice with Nakayama protects against this type. American workers (Paterson *et al.*, 1952) who noticed this phenomenon with a strain they isolated in Malaya suggested that this failure to protect against the Nakayama strain could either indicate a slight antigenic difference or be due to the fact that the Nakayama strain was better adapted to the mouse. We have observed this difference so constantly with G.H.1/52, G.H.3/52, M.7/52 and M.1/53 that we feel it must be due to a minor antigenic difference as most of the strains have now been passed through mice many times.

In cross neutralisation tests these differences, even the greater differences shown by the Muar and Tengah strains, are to some extent reduced. Immunisation of rabbits to obtain antisera means multiple inoculations of virus suspensions and such sera tend to be less specific. On the other hand vaccination of mice for cross resistance tests and for the production of sera to be used in complement fixation involves fewer inoculations and the antibodies produced are likely to be of more specific character.

The simplest antigenic composition of the three serological types that will fit our findings can be represented diagrammatically in the Figure.

Further investigation of the antigenic structure of these strains is being carried out by the micro-complement fixation technique, described by Fulton and Dumbell (1949).

SUMMARY.

Six strains of Japanese B encephalitis virus isolated from the brains of fatal human cases occurring in Malaya have been investigated by serological methods.

Cross neutralisation tests with immune sera using intracerebral inoculation in mice demonstrate that all are closely related.

Complement fixation and cross resistance studies showed that the viruses could be subdivided into three serological types.

One type differs more markedly than the others and the advisability of referring to this as Malaya encephalitis virus is discussed but discarded in favour of regarding it as a serological type of Japanese B virus.

The possible antigenic composition of the three serological types is suggested.

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THE OXYGEN CONSUMPTION OF THE BLOOD IN EXPERIMENTAL LEAD POISONING

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IT was shown by McFadzean and Davis (1949) that the primary haematological effect of lead poisoning is on haemoglobinating normoblasts in the bone marrow. They found that these cells, and non-nucleated erythrocytes in the bone marrow, showed cytoplasmic stippling even before stippled cells were seen in the peripheral blood. In lead-poisoned guinea-pigs they found both punctate basophilia as seen in human cases of chronic lead poisoning, and erythrocytes containing fewer, large, irregular inclusions. These larger stipples gave a positive reaction for iron more often than the smaller ones and were associated with appearances of impaired haemoglobination. Further experiments by McFadzean and Davis, Pirrie (1952), and Baikie (1954) have shown that the anaemia of chronic lead poisoning has the characteristics of a haemolytic anaemia. The increased rate of red cell destruction would appear to be due to the elimination of these stippled, and presumably defective red cells, some of which show evidence of malhaemoglobination and hypochromia. In a series of experiments with lead-poisoned guinea-pigs the number of cells showing reticulum when stained supravitally with cresyl blue has been found to be roughly twice the number of stipple cells in films of the same blood stained with Leishman diluted with buffer of pH 7·2 (Baikie and Pirrie, 1954). Whitby and Britton (1933) studied the effects of acute lead poisoning and phenylhydrazine poisoning in rabbits and showed by various staining techniques that stippling and polychromasia are both manifestations of reticulation. They did not regard the stippled red cell as damaged or defective and did not describe hypochromia or malhaemoglobination.

A functional characteristic of the reticulocyte is its higher oxygen consumption. Mature red cells have a negligible oxygen consumption (Warburg, 1909) but nucleated red cells and young non-nucleated cells, including those distinguished by polychromasia or the presence of reticulum, have a higher oxygen consumption (Morawitz, 1909; Harrop, 1919). As young red cells having similar basophilic material to ordinary reticulocytes, stippled cells might be expected to have the same high oxygen consumption. On the other hand, since their haemoglobination is imperfect, stipple cells might be expected to show other evidence of impaired metabolism, such as a lower oxygen consumption than other reticulocytes. Since there have been no published observations on the oxygen consumption of stippled erythrocytes this investigation was undertaken to see if in that respect they differ from other cells which show reticulum when stained with cresyl blue.

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METHODS.

Guinea-pigs were poisoned with lead by the oral administration of a solution of lead nitrate in doses varying from 75 mg. to 150 mg. daily. Reticulocytosis was produced in other guinea-pigs either by the repeated withdrawal of blood by sterile cardiac puncture, or by the administration of anti-guinea-pig red cell serum resulting in a haemolytic anaemia. Anti-guinea-pig red cell serum was prepared in rabbits by a modification of the method of Dameshek, Schwartz and Gross (1938).

Blood for study of oxygen consumption was obtained by cardiac puncture and subsequently handled with full sterile precautions. The blood was defibrinated and oxygenated by shaking for 15 min. in a flask containing glass beads. Clot was removed by filtration through glass wool. The filtrate was apportioned between 2 bijou bottles; one portion was kept for immediate determination of oxygen content and for the other haematological observations, while the second was placed in an incubator at 37° after the addition of a thick layer of sterile liquid paraffin. In the earlier experiments of this study the blood samples were incubated for 5 or 6 hr. At the end of such a period samples with high reticulocyte counts had consumed all the available oxygen. Since the time taken to exhaust the available oxygen was unknown these results were discarded and in the later experiments the blood was incubated for $3\frac{1}{2}$ or 4 hr. The oxygen content of the blood was determined in 1 ml. samples using the Van Slyke manometric apparatus, oxygen consumption being the difference between the oxygen content before and after incubation. Determinations of the packed cell volume, red cell count, and the total nucleated cell count were all carried out in duplicate. The number of reticulocytes per 100 red cells was found by counting 1000 cells in wet preparations stained with cresyl blue.

RESULTS.

Oxygen consumption is expressed as volumes per cent per hour divided by the packed cell volume. Results are shown in full in Tables I and II. In the Figure oxygen consumption is plotted against the reticulocyte or stipple cell percentage. It will be seen that the oxygen consumption of blood is directly proportional to the percentage of reticulocytes irrespective of whether these occur in lead poisoning or during active regeneration after bleeding or haemolytic anaemia. This correlation between oxygen consumption and reticulocyte or stipple cell count is fairly

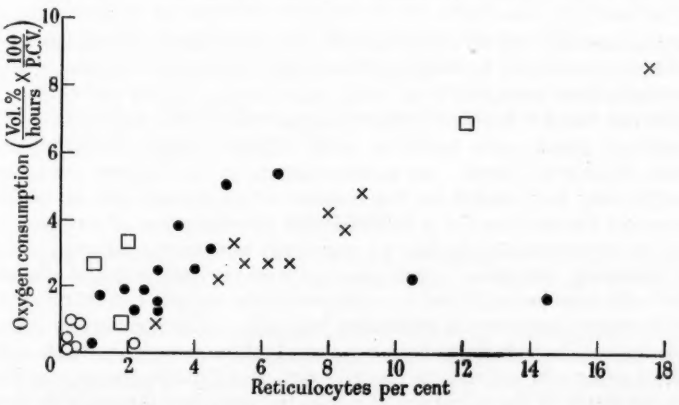

FIGURE.—Oxygen consumption and reticulocyte percentage of guinea-pig whole blood. ○ = normal; ● = lead-poisoned; × = post-haemorrhagic; □ = post-haemolytic.

TABLE I.—*The Oxygen Consumption of the Blood and other Haematological Values of Normal and Lead-Poisoned Guinea-Pigs.*

No.	Condition	O_2 capacity vol. %.	Hb. g. %.	P.C.V. %.	R.B.C. mill./ c.mm.	Retics. %.	Nuc. cells thous.	Nuc. Non-nuc.	O_2 consumption. Vol. %.	O_2 consumption. Vol. % / Hours × 100/P.C.V.
1	Normal	24·4	17·7	60·5	6·88	0·3	6·0	1/1100	0·5	0·17
3	,,	20·2	14·9	53·5	5·82	2·2	7·4	1/800	1·2	0·37
5	,,	21·4	16·1	54	7·41	0·2	4·6	1/1600	*nil*	*nil*
7	,,	21·5	16·0	54	5·64	0·5	10·4	1/500	3·2	0·99
8	,,	17·0	12·9	37·5	4·34	0·2	3·6	1/1200	1·0	0·44
11	,,	25·8	18·9	59	6·80	0·3	7·0	1/1000	3·5	0·99
4	Lead-poisoned	15·8	11·5	37·5	4·71	10·5	5·4	1/900	5·0	2·22
10	,, ,,	6·1	4·5	12·5	1·60	14·5	6·4	1/300	1·2	1·60
17	,, ,,	10·0	7·3	21·5	3·63	5·0	3·8	1/1000	6·5	5·04
28	,, ,,	17·5	9·6	31·5	4·03	6·5	5·6	1/700	5·5	5·37
31	,, ,,	19·5	14·3	44	5·14	3·8	9·6	1/500	5·0	3·50
33	,, ,,	19·2	14·1	47·5	5·33	1·0	5·6	1/1000	0·2	0·32
34	,, ,,	18·8	13·8	35·5	4·88	1·2	8·8	1/600	2·0	1·74
35	,, ,,	19·0	14·2	46	5·02	2·9	8·5	1/600	2·2	1·48
36	,, ,,	21·2	15·8	46	5·09	1·9	5·7	1/900	2·8	1·87
37	,, ,,	21·8	16·3	51	5·46	2·9	5·2	1/1100	2·2	1·33
38	,, ,,	20·5	15·3	45·5	4·97	2·5	5·0	1/1000	2·8	1·89
39	,, ,,	21·4	16·0	50·5	5·42	2·2	3·1	1/1700	2·2	1·34
40	,, ,,	17·1	12·7	44	4·64	4·5	6·8	1/700	4·4	3·08
41	,, ,,	20·0	15·0	48	5·04	2·9	2·9	1/1700	3·8	2·44
42	,, ,,	19·0	14·2	43·5	4·80	4·0	5·2	1/900	3·5	2·48

TABLE II.—*The Oxygen Consumption of the Blood and other Haematological Values of Guinea-Pigs after Repeated Bleeding or Experimental Haemolytic Anaemia.*

No.	Condition.	O_2 capacity vol. %.	Hb. g. %.	P.C.V. %.	R.B.C. mill./ c.mm.	Retics. %.	Nuc. cells. thous.	Nuc. Non-nuc.	O_2 consumption. Vol. %.	O_2 consumption. Vol. % / Hours × 100/P.C.V.
14	Post-haemolytic	10·2	7·9	23·5	2·33	12·1	8·0	1/300	9·8	6·99
26	,, ,,	17·6	13·0	40·5	4·28	2·0	16·2	1/300	4·4	3·34
27	,, ,,	20·7	15·2	45·5	5·31	1·8	4·8	1/1100	1·3	0·87
30	,, ,,	15·8	11·6	32	3·56	1·0	19·0	1/200	2·2	2·60
2	Post-haemorrhagic	13·8	10·1	29·5	3·90	6·2	6·2	1/600	4·0	2·66
6	,, ,,	23·1	17·3	51	5·61	2·8	3·8	1/1500	2·6	0·85
9	,, ,,	19·0	13·9	42	4·41	6·8	1·4	1/3200	7·0	2·77
12	,, ,,	16·2	11·9	36·5	4·65	8·0	6·8	1/700	9·2	4·20
18	,, ,,	20·0	14·8	44·5	5·51	4·7	6·0	1/900	5·9	2·20
19	,, ,,	19·8	14·5	45·5	4·09	5·5	6·0	1/700	7·5	2·74
23	,, ,,	12·0	8·8	31·5	4·11	17·5	9·6	1/400	8·8	8·60
25	,, ,,	17·5	12·9	41	5·11	5·2	7·8	1/700	4·5	3·38
29	,, ,,	9·4	6·9	20·5	2·51	8·5	3·2	1/800	2·5	3·75
32	,, ,,	17·5	12·9	38·5	4·14	9·0	5·8	1/700	6·0	4·80

consistently observed. In 35 experiments only 2 notable exceptions were seen. The blood of animals 4 and 10, both lead-poisoned, had lower oxygen consumption than would have been expected from their reticulocyte-stipple cell counts. In no other respect did these 2 animals differ from the rest of the lead-poisoned guinea-pigs. Nucleated blood cells have an even higher oxygen consumption than reticulocytes (Morawitz, 1909). In measurements of the oxygen consumption of whole blood it may be possible for the number of nucleated cells to be increased so as to account themselves for a considerable consumption of oxygen. On the other hand, in experiments similar to our own, but in the anaemia of phenyl-hydrazine poisoning, Morawitz (1909) showed that variations in the concentration of nucleated cells may be neglected in considering the oxygen consumption of blood containing increased numbers of immature red cells. Furthermore, in the present experiments, as can be seen from Tables I and II, there were no notable differences between the 4 groups of animals in the proportions of nucleated to non-nucleated cells. Consequently, if the numbers of nucleated cells had appreciably influenced the oxygen consumption of the blood this influence would have been no more marked in one group than in another. The absence of any correlation between

oxygen consumption and total nucleated cell count makes such an effect most improbable.

DISCUSSION.

Our finding that the reticulocyte-stipple cells of lead poisoning respire as actively as other reticulocytes is consistent with stipple cells being immature red cells. Previously, the belief that stipple cells are young and not senescent cells was dependent on their having basophilic material in the cytoplasm in common with reticulocytes and polychromatic red cells (Whitby and Britton, 1933). More recently, this view has received support from the demonstration by McFadzean and Davis (1949) that before stipple cells are seen in the peripheral blood they are to be found in bone marrow. It is now shown that stipple cells share at least one important functional characteristic with normal reticulocytes. In whatever way they are damaged by lead it does not apparently interfere with what is almost certainly an intracellular oxidative process productive of energy.

On teleological grounds alone it is an attractive suggestion that the presence of reticulum in immature red cells is associated with the formation of haemoglobin. Seip (1953) points out that this suggestion was first made by Giglio-Tos as long ago as 1898. It has recently been placed on firmer ground by the demonstration by Walsh, Thomas, Chow, Fluharty and Finch (1949) that reticulocytes, but not adult erythrocytes, can both take up iron and synthesize haem *in vitro*, while Douglas and Dacie (1953) have correlated the occurrence of iron-containing granules in red cells with the immaturity of these cells. Similarly, London, Shemin and Rittenberg (1950) also found reticulocytes able to synthesize haem and correlated the cessation of this activity with the disappearance of reticulum. It seems most likely that the active oxidative process within reticulocytes subserves this continuing haemoglobination, probably by providing the chemical energy for the synthesis of haem. The finding that this oxidative process is quantitatively as active in stipple cells as in normal reticulocytes is consistent with the hypothesis first advanced by Rimington (1938) that lead interferes with the elaboration of haem by blocking an enzymic process in its synthesis. If lead inflicted less specific damage on the developing red cell the normal rate of respiration might well be diminished or absent. As it is, it must remain a matter of conjecture why defective haemoglobination, whether in lead poisoning or in certain human cases of haemolytic anaemia (McFadzean and Davis, 1947) with similar red cell changes, is associated with decreased red cell life.

SUMMARY.

The oxygen consumption of the blood of lead-poisoned guinea-pigs has been studied. It is much higher than the oxygen consumption of the blood of normal animals.

The rate of oxygen consumption of the blood of lead-poisoned animals is comparable with the high oxygen consumption of the blood of animals with reticulocytosis after bleeding or haemolytic anaemia, being roughly proportional to the reticulocyte or reticulocyte-stipple cell content of the blood in all three conditions.

This finding is discussed in relation to the known defect of haemoglobination in lead poisoning.

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THE ANTIGENS PRODUCED *IN VITRO* BY *MYCOBACTERIUM TUBERCULOSIS*, WITH SOME OBSERVATIONS ON THE FREQUENCY OF ANAPHYLACTIC SENSITIZATION DURING THE COURSE OF EXPERIMENTAL TUBERCULOSIS.

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In the normal guinea-pig tuberculo-protein behaves like other soluble antigens and gives rise to anaphylactic hypersensitivity, in contrast to the delayed form of allergy which tuberculo-protein evokes during the course of tuberculosis. Tuberculous animals may exhibit both these types of hypersensitivity, but the occurrence of the anaphylactic form is usually regarded as inconstant (Lewis and Seibert, 1931; Corper, 1940; Rich 1951). The factors which determine the differing immunological response when the antigen is released during the course of tuberculous infection are not fully understood, but it has been shown experimentally by Raffel and his collaborators (Raffel, 1948; Raffel, Arnaud, Dukes and Huang, 1949) that the tubercle bacillary wax may modify the type of hypersensitivity evoked by an antigen with which it is incorporated. Nevertheless, it is doubtful whether this provides a complete explanation for the development of delayed-type hypersensitivity, since this phenomenon is a characteristic of several diseases apart from tuberculosis, in many of which the causal agent is not characterized by a high wax content (*e.g.*, variola). The finding of Birkhaug (1941) that anaphylactic hypersensitivity to unheated tuberculo-protein is absent in the earlier stages of tuberculosis, but develops regularly after 5–6 months' infection, is therefore of considerable immunological interest, and the work described in this paper was carried out in an attempt to confirm his finding.

For this purpose the Dale reaction provides a delicate technique for the detection of anaphylactic sensitization. As a preliminary, it was decided to study the antigens produced by the tubercle bacillus, since information on this subject might permit a more detailed appreciation of their significance as sources of anaphylactic hypersensitivity in tuberculosis.

MATERIALS AND METHODS.

Experimental animals—Guinea-pigs, usually males weighing 400–600 g. were obtained from the State Serum Institute, Copenhagen. They received a diet of turnips, oats, hay and, during the summer months, grass.

Method of Antigen Analysis of Culture Filtrates.

Principle of the method.

The method of antigenic analysis depended on the fact that when a guinea-pig is sensitized with a mixture of antigens (such as exist in tubercle bacillary culture filtrates), its

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isolated smooth muscle, suspended in a Dale bath, will exhibit a specific anaphylactic contraction followed by desensitization when each of the components of the original antigenic mixture is added in turn. It is thus possible to study the antigenic relationships of different protein and polysaccharide fractions prepared from tubercle bacillary culture filtrates.

Sensitization procedures.

Sauton medium, in which virulent human tubercle bacilli (strain E5) had been growing for 6 weeks, was passed through a Jena G5 glass filter to remove the organisms. The medium at the end of the growth period had a reaction of approximately pH 5. The filtrate was treated as follows, being either neutralized with dilute ammonia, or neutralized with dilute ammonia, and then heated in a water bath at 100° for 1 hr., or heated for 1 hr. at 100° without previous neutralization. Heating of the unneutralized medium rapidly causes flocculation of some of the protein. The flocculate was removed by filtration.

Concentrates (10–25-fold) of the respective filtrates were also prepared by pressure dialysis through 9 per cent collodion (Parlodion) membranes.

Groups of guinea-pigs were sensitized with filtrates or concentrates. With filtrates, 4 doses (total volume 4–8 ml.) were given during 4–5 weeks, while the animals sensitized with concentrates received 4–5 doses (total volume 1·0—1·5 ml.) during 4–6 weeks. The first 2 doses were given intraperitoneally and the remainder subcutaneously. The experiments were usually carried out within 3–4 weeks of the last sensitizing injection and all the animals were found to be highly sensitized.

Antigenic fractions.

I am indebted to Fru L. Lundberg for samples of the following materials and for the details of their preparation.

Tuberculo-polysaccharides.—These were either obtained from the concentrate of unheated medium by ethanol precipitation after previous removal of the proteins, or prepared from a buffer phosphate extract (pH 8) of heat-killed tubercle bacilli (strain E5). After concentration *in vacuo* and removal of proteins, the polysaccharides were precipitated with ethanol.

Polysaccharides thus obtained were re-dissolved in water and re-precipitated 3 times. The dose employed in the experiments was 0·5 mg. dissolved in 0·5 ml. water.

P.P.D.—Prepared by the method of Jensen, Bindslev, Møller, Hansen and Lind (1938). The test dose was 3 mg. dissolved in 0·5 ml. of Sørensen's 0·06 M phosphate buffer (pH 7·38).

Other protein fractions.—These consisted of the proteins precipitated from the concentrate of unheated medium (unheated protein), and the concentrate of medium heated at pH 7 (heated protein). The proteins were obtained by precipitation at pH 7, using a modification of the method of Seibert and Glenn (1941). The unheated protein was obtained by precipitation with 75 per cent saturated ammonium sulphate, while for the heated protein, 50 or 75 per cent saturated ammonium sulphate was employed. The proteins were re-precipitated 6 times. The test dose of each was I mg. dissolved in 0·25 ml. water (unheated protein) or 0·25 ml. 0·06 M phosphate buffer (heated protein). Polysaccharide was present in undetermined amounts in both these protein fractions and also in P.P.D., and the test doses contained sufficient to elicit typical anaphylactic contractions in polysaccharide-sensitized animals.

Method of performing the Schultz-Dale test.

Guinea-pigs were killed by a blow on the head and the terminal part of the small intestine was rapidly transferred to a dish of warm Tyrode's solution, where it was gently washed free of its contents. A strip about 4 cm. long was then suspended in a Dale bath of 23 ml. capacity containing constantly oxygenated Tyrode's solution at 37° and the movements of the intestine recorded kymographically. The test substances were injected into the bath from graduated syringes, and after each addition, the movements of the gut were recorded for at least 2 min. If a contraction occurred, it was allowed to continue until the character of the response had become clearly defined and the gut was beginning to relax. The bath was then washed out and refilled, and when the gut had completely relaxed, a second dose of the same material was usually added to confirm that desensitization had taken place. (Occasionally, to conserve available supplies of the test substances, this second dose was omitted.) The response of the strip of intestine to a standard dose of 0·2 μg. histamine acid phosphate was observed before and after each series of tests. The time was recorded throughout the experiments at 10-sec. intervals.

Infection of guinea-pigs with tuberculosis.

The following strains of tubercle bacilli were employed :

B.C.G.—The standard strain of the State Serum Institute, Copenhagen, grown on Sauton medium, and injected intraperitoneally or intramuscularly in 10 mg. (semi-dry weight) doses. The bacilli were washed 3–4 times in normal saline to remove residual medium protein which might otherwise sensitize the animals.

E5.—A highly virulent strain of human type. The following doses were used: 0·000001 mg.; 0·0001 mg.; 0·001 mg. in paraffin oil; 2mg. (heat-killed) in paraffin oil.

H37Rv.—National Collection of Type Cultures No. 7416. It was used in doses of 0·01 mg. and 1·0 mg.

The E5 and H37Rv strains were cultivated on Löwenstein's medium. Except where otherwise stated, the infective suspensions were prepared in normal saline and injected intramuscularly into the right thigh. The guinea pigs were killed 4—32 weeks after inoculation.

RESULTS.

Effect of antigenic fractions on non-sensitized intestine.

None of the antigenic fractions produced any effect on the non-sensitized intestine comparable to a typical anaphylactic contraction, although the unheated protein and the concentrates sometimes tended slightly to stimulate spontaneous intestinal activity. The addition of P.P.D. to the bath was followed after a few seconds by relaxation of the gut and a marked loss of spontaneous activity, which persisted until the P.P.D. was washed out; a similar effect was occasionally observed with the other fractions. (This did not inhibit the specific anaphylactic contraction in the sensitized intestine, since the initial relaxation was quickly followed by the typical anaphylactic response after the usual latent period.)

Character of the anaphylactic response.

The anaphylactic contractions were preceded by a latent period of inactivity varying from 5–40 sec. When the gut was highly sensitized, there was a short latent period and a rapid contraction followed by gradual relaxation during the succeeding 5–10 min. With less intense sensitization, the latent period was longer and the contraction slower. With intestinal strips giving responses to several antigenic fractions in turn, the later contractions tended to be slightly weaker than the earlier.

Order of addition of antigens to the bath.

The most suitable sequence in which to test the antigenic fractions on the intestine was studied in some preliminary experiments using intestine from guinea-pigs sensitized with unheated concentrate. The materials were tested in 3 alternative orders, using different strips of gut from the same animal. The findings are shown in Table I. All fractions giving positive reactions were re-tested a second time after the gut had completely relaxed and had been washed. In no instance did the second dose evoke any evidence of residual sensitization.

Exp. I shows that unheated protein desensitizes the gut to both the other protein fractions and must therefore contain the antigens present in them. Exp. II shows that exposure of the intestine to heated protein desensitizes the gut to P.P.D. but not to unheated protein. Evidently therefore, heated protein contains the antigens present in P.P.D., but lacks an antigenic component present in

TABLE I.—*Anaphylactic Responses Produced by Antigenic Fractions Tested in Various Orders on Intestine Sensitized to Unheated Concentrate.*

Order of testing.	Experiment. I.		Experiment. II.		Experiment. III.	
1	—		—		Polysaccharide	+
2	Unheated protein	+	Heated protein	+	P.P.D.	+
3	P.P.D.	−	P.P.D.	−	Heated protein	+
4	Heated protein	−	Unheated protein	+	Unheated protein	+

+ contraction ; − no contraction.

unheated protein. In Exp. III, the intestine, after preliminary desensitization to polysaccharide, reacted in turn to P.P.D., heated protein and unheated protein. This confirms the previous findings and also indicates the presence in heated protein of an antigen absent from P.P.D. Evidently, therefore, unheated protein contains, in addition to polysaccharide, 3 distinct antigenic components, 2 of which are present in heated protein, and one in P.P.D. The most suitable order in which to add the various fractions to the organ bath when testing sensitized intestines is that in Exp. III, since this permits an exact appreciation of the responses to the individual antigenic components.

Antigenic analysis of unheated and heated culture filtrates.

The intestines of guinea-pigs, sensitized with the various filtrate preparations (or their respective concentrates) reacted to the different antigenic fractions as shown in Table II. No difference was observed between the results of sensitizing with media and with concentrates. In all three groups, some irregularity was noted in the degree of sensitization to polysaccharide, but in each of the groups some of the animals showed strong reactions to it. It was therefore accepted that antigenic polysaccharide could persist in the medium after all 3 methods of treatment.

TABLE II.—*Antigenic Analysis of Unheated and Heated Culture Filtrates.*

Sensitizing filtrate or concentrate.	Anaphylactic response. Polysaccharide.	P.P.D.	Heated protein.	Unheated protein.	Respective concentrate.
Unheated	+	+	+	+	−
Heated at pH 7	+	+	+	−	−
Heated at natural pH	+	+	−*	−	−

* One sample of medium produced some sensitization to this fraction.

These findings indicate that the polysaccharide and protein fractions together incorporate all the antigenic constituents of the original filtrate preparations. This conclusion should, perhaps, be qualified by the evidence afforded by some of the experiments with tuberculous animals (*vide infra*). The results shown in Table II indicate that all the sensitizing materials contained some antigenic polysaccharide, accompanied in the case of the unheated filtrates by 3 protein antigens. The filtrates heated at pH 7 or at their unadjusted pH contained only 2 or 1 protein antigen respectively.

Incidence of anaphylactic hypersensitivity in tuberculous animals.

With the information derived from the foregoing study of the antigens produced by the tubercle bacillus *in vitro*, a survey was carried out to determine the frequency with which anaphylactic hypersensitivity to these antigens is engendered during the course of tuberculosis. A strip of gut from each tuberculous animal was tested in turn against polysaccharide, P.P.D. and unheated protein; in many instances, however, (including all those in which the previous fractions had elicited positive responses), a separate piece of intestine was used for testing the unheated concentrate. The results are summarized in Table III.

TABLE III.—*Incidence of Anaphylactic Hypersensitivity to Tubercle Bacillary Antigens during Tuberculous Infection.*

Group.	Time infected.	Number in group.	Number hypersensitive to: Polysaccharide 1 mg.	P.P.D. 3 mg	Unheated protein 1 mg.	Unheated conc. 0·25 ml.
(A) B.C.G. i/m	<20 weeks	12	2(4)	1	0(4)	2
	20 weeks or more	5	0(1)	0(1)	0(1)	0
(B) B.C.G. i/p	<20 weeks	2	0	0	0	0
	20 weeks or more	4	0	0	2	2
(C) E5 10^{-6} mg.	<20 weeks	2	0	0	0	0
	20 weeks or more	3	1	0	0	1
(D) E5 10^{-4} mg.	<20 weeks	6	0(5)	0	0(4)	0
	20 weeks or more	2	0	0	0	0
(E) E5 10^{-3} mg. in oil	<20 weeks	5	0(4)	0(1)	1(4)	2
	20 weeks or more	0	—	—	—	—
(F) E5 2 mg. (dead) in oil	<20 weeks	1	1	0	0	1
	20 weeks or more	2	1	0	0	2
(G) E5 aerosol of 0·05 mg./ml. suspension	>20 weeks	2	1	1	1	2
			same animal			
(H) H37Rv 10^{-3} mg.	<20 weeks	7	0(2)	0	0(1)	2
	20 weeks or more	4	0	0	0	0
(I) H37Rv 1 mg.	<20 weeks	7	0(5)	0	0(1)	0

The figures in parenthesis indicate the number of each group not tested with a particular fraction.

Anaphylactic hypersensitivity to any of the antigens occurred only irregularly and rather infrequently. It showed no consistent or significant predilection for the later stages of the disease. Thus, taking all groups into consideration, of the 42 guinea-pigs which had been infected for less than 20 weeks, 5 animals displayed anaphylactic sensitization to either polysaccharide or protein. Of the animals infected for 20 weeks or more, 5 out of 22 were sensitized in this way. The difference, however, is not significant statistically ($\chi^2 = 1{\cdot}28$; $P = > 0{\cdot}2$).

It will be seen from Table III that some animals were hypersensitive to concentrate, but did not react to polysaccharide or protein. These latter substances, if tested on the gut before the concentrate, apparently desensitized it, since a subsequent dose of concentrate would fail to elicit any contraction. This type of hypersensitivity could only be detected by exposing the gut to the concentrate before the other fractions. A typical example of this type of response is illustrated in the Figure.

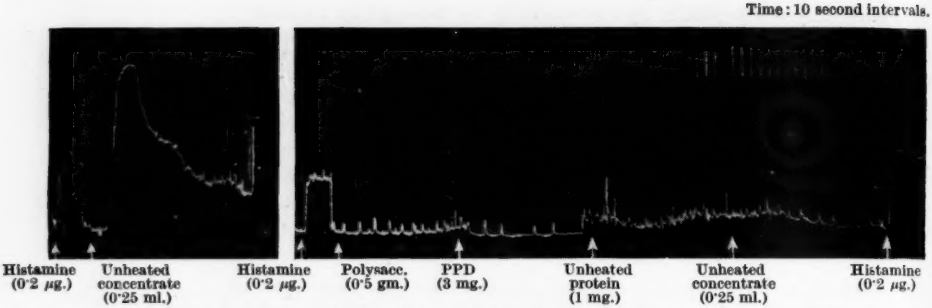

FIGURE.—Tracings of guinea-pig intestinal contractions, showing a reaction to concentrate but not to polysaccharide or protein. It will be observed that the gut is desensitized to concentrate by previous treatment with polysaccharide and protein.

As might be expected, in virtue of its polysaccharide and protein content, unheated concentrate regularly produced contractions in the intestines of the polysaccharide- and protein-sensitized guinea-pigs provided it was tested on a separate piece of gut.

DISCUSSION.

The cross-anaphylaxis technique employed in the present study provides a method of distinguishing some of the various antigenic and haptenic constituents of culture filtrates of *Mycobacterium tuberculosis*. The results described indicate the presence in such filtrates of at least four components. One of these is a polysaccharide and the remaining three are apparently proteins. It is quite possible, and indeed probable, that some of these entities are not homogeneous and each may well be a complex of several antigens and haptenes. However, in the absence of data on this point, it is convenient to refer to the various components as if each were a single pure antigen.

Unheated filtrates contained polysaccharide and all three protein antigens, while in medium heated at pH 7, only polysaccharide and two proteins were evident. The filtrate heated at its natural pH (about 5) and then filtered to remove flocculated protein, contained only polysaccharide and a protein antigen identifiable with that in the P.P.D. Thus, by neutralizing the culture filtrate before heating, a protein antigen was retained in solution which would otherwise have been eliminated by heat coagulation. It is clear, therefore, that the pH of the culture filtrate at the time of heating has a bearing on its subsequent content of protein antigens. Since commercial P.P.D. is prepared from medium which has been heated and filtered at its natural pH, its antigenic composition is likely to vary according to the pH of the original batch of medium from which it was derived. This factor might explain the observation (Table II) that one batch of medium heated under these conditions conferred slight sensitivity to heated protein as well as P.P.D. From the results of some of the experiments with tuberculous guinea-pigs, it is evident, however, that unheated concentrate contains one or more additional antigens besides those already characterized in the earlier

experiments. The intestines of some tuberculous guinea-pigs were hypersensitive to unheated concentrate, though not to polysaccharide or the protein preparations. Previous exposure to the two latter substances apparently desensitized the gut to the component in the unheated concentrate. This effect is perhaps attributable to the presence of traces of this unknown antigen in one or both of these preparations, but in quantities too small to elicit an anaphylactic contraction. The chemical nature of the antigen concerned, however, remains unknown.

The frequency with which these various tubercle bacillary antigens gave rise to anaphylactic hypersensitivity during the course of tuberculosis was investigated in groups of tuberculous and B.C.G.-injected guinea-pigs. Birkhaug (1941) contended that the development of anaphylactic hypersensitivity to unheated tuberculo-protein was a regular feature of the later stages of tuberculosis. The intravenous injection technique which he employed in his experiments to demonstrate the presence of anaphylactic sensitization did not permit any differentiation between sensitization to polysaccharide or proteins. In the present investigation it was not possible to confirm Birkhaug's observations. Anaphylactic sensitization occurred only occasionally, and in only two groups (B and C) did it show any predilection for the later stages of the disease. In one of these groups (C), polysaccharide was responsible for the sensitization, while in the other (B), protein was concerned. In those groups that remained, and where sufficient animals were examined at all stages of the disease to justify a conclusion (groups A, D, H), sensitization to one or other of the antigens occurred irregularly after various durations of infection, or was completely absent. There was no statistically valid difference between the incidence of sensitization in guinea-pigs infected for less than 20 weeks and those infected for this period or longer.

SUMMARY.

By the technique of cross-anaphylaxis unheated tubercle bacillary culture filtrates have been found to contain a polysaccharide antigen and three antigens associated with the proteins. In filtrates heated at pH 7, polysaccharide and only two protein antigens could be detected. In filtrates heated at their natural pH (about 5), polysaccharide and a protein antigen identical with that in P.P.D. were identified. Experiments with tuberculous guinea-pigs suggest the existence of a further antigen which is present particularly in unheated concentrates. The chemical nature of this substance is unknown.

Anaphylactic hypersensitivity to polysaccharide or protein fractions occurred irregularly or infrequently at various stages of tuberculosis. Its occurrence showed no constant predilection for the later stages of the disease.

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FOOD AS A VEHICLE OF INFECTION: THE EFFECT OF VARIATIONS IN THE DIET ON THE INDUCTION OF *SALMONELLA GALLINARUM* INFECTION.

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Most studies on nutritional aspects of bacterial infection have been concerned with the effect different constituents of the diet may have in modifying the resistance of the host to the activities of the parasite and thus influencing the outcome of the infection. The influence of the character of the food consumed at the actual time of ingestion of pathogenic bacteria on the defence-mechanisms of the alimentary tract has received much less attention. Some of these mechanisms, such as the barrier presented by gastric juice, are well known and have been studied, notably by Arnold and his co-workers (Arnold, 1926, 1927*a*, *b*, 1928, 1929; Arnold and Brody, 1926*a*, *b*, 1927). The *in vitro* effect of gastric juice on pathogenic bacteria that enter the tissues *via* the intestinal tract has also been investigated by Garrod (1937, 1939).

During recent studies in which attempts were made to develop a standard method of infecting chickens by mouth with *Salmonella gallinarum*, a higher infection rate was obtained in chickens fed a commercial mash at the time of administering *Salm. gallinarum* than was obtained in chickens fed whole wheat. Further work showed that this was associated with a much greater destruction of *Salm. gallinarum* in the alimentary tract of wheat-fed than in mash-fed chickens. It was therefore decided to investigate the subject more fully, a study which was aided by the fact that the stomach of the chicken can be considered to consist of 3 parts, the crop in which recently ingested food is stored, the proventriculus which secretes the gastric juice, and the extremely muscular gizzard in which food material is well mixed with the gastric juice and ground to a relatively fine consistency before being released into the duodenum.

MATERIALS AND METHODS.

Chickens.

Sex-linked Brown Leghorn × Barred Rock cockerels, aged approximately 9 weeks, from a salmonella-free flock were used. They were fed a mixed diet of mash and whole wheat.

Food materials.

The mash consisted of the following ingredients: wheat meal 12·5 per cent, barley meal 12·0 per cent, oat meal 16·0 per cent, pea meal 3·0 per cent, maize meal 15·0 per cent, middlings 15·0 per cent, maize gluten feed 5·0 per cent, grass meal 7·5 per cent, mineral mixture 1·25 per cent, synthetic vitamin D supplement 0·25 per cent, white fish meal 10·0 per cent, dried yeast 2·5 per cent.

The approximate formula of the fish meal was: protein 66 per cent, oil 3·5 per cent, soluble carbohydrates 10·5 per cent, ash 20 per cent.

The whole wheat was grown locally. When used as a meal it was ground either to the consistency of domestic flour (fine wheat meal) or less finely (coarse wheat meal).

The alkali employed was a powder of the following composition : powdered chalk 40 per cent, colloidal kaolin 43 per cent, magnesium trisilicate 17 per cent.

Commercial casein was used consisting of 99 per cent protein. Chickens fasted for 18 hr. were used in experiments in which the effect of different diets was to be studied.

Salm. gallinarum *culture.*

Unless otherwise stated, one strain of *Salm. gallinarum*, strain 9, was used for all experiments. It was maintained without subcultivation on Dorset's egg medium at 4°. When required for use, it was subcultivated into nutrient broth and incubated for 18 hr. at 37°. The same batch of broth was used throughout.

Determination of the number of bacteria used in infection experiments.

Since it was necessary to give chickens known amounts of viable *Salm. gallinarum*, the nephelometer reading of an 18-hr. broth culture of strain 9 was first determined and a viable count carried out by the method of Miles and Misra (1938), using nutrient agar. Cultures used in subsequent experiments were adjusted after 18 hr. incubation to give the same reading on the nephelometer, and diluted according to the dose required. A viable count was carried out at the same time so that the estimated count could be confirmed on the following day.

Estimation of the numbers of Salm. gallinarum *in the contents of the alimentary tract.*

This was carried out by a modification of the method of Miles and Misra. Approximately 1 ml. of the contents of the organ under study were removed from the chicken as soon as possible after death and added to 9 ml. of sterile distilled water containing a little alkali to neutralize any acid present, and thus to prevent any further destruction of bacteria. Additional dilutions were then made and counts performed on desoxycholate-citrate-agar (D.C.S.S.) containing 1 per cent each of sucrose and salicin (Williams Smith and Buxton, 1951). Counts of cultures of *Salm. gallinarum* on this medium and on nutrient agar gave similar results.

Determination of pH of the contents of the alimentary tract.

As soon as possible after chickens were killed, the contents of the organs under study were removed, a small amount of distilled water added if necessary, and the pH determined by means of a Cambridge pH indicator.

Technique of infection experiments.

In experiments designed to compare the infection rates obtained as a result of administering *Salm. gallinarum* in different foods to groups of chickens, each chicken was kept in a separate battery cage and precautions taken to prevent cross-infection, no evidence of which was ever noticed. After first fasting for 18 hr., each chicken was given 15 g. of the food under study and, approximately 1 hr. later, the desired dose of *Salm. gallinarum* was mixed thoroughly with 30 g. of the same type of food, water being added when necessary for satisfactory mixing. This amount of food was usually consumed within 2 hr., when the food container was removed. The same type of food in a sterile container was then given *ad lib.* for a further 18 hr. when it was replaced by the usual laboratory diet of mash and whole wheat. Each experiment lasted 3 weeks and the number of chickens dying in each group during this period was noted. Previous studies had shown that only a small proportion of infected chickens died after this time. The survivors were submitted to a plate agglutination test using whole blood and a stained antigen prepared from the same strain of *Salm. gallinarum* as was used in the experiments (Gordon and Brander, 1942). Rectal swabs were also examined. These were incubated at 37° for 24 hr. in selenite-F medium (Hobbs and Allison, 1945) and streaked on plates of D.C.S.S.'medium. The plates were incubated at 37° for 24 hr. and examined for the presence of *Salm. gallinarum*. The chickens were then killed and all those either showing lesions characteristic of infection, or positive by agglutination or cultural tests were recorded as being infected ; with very few exceptions, the chickens were either positive or negative to all 3 tests.

RESULTS.

The effect of different diets on the pH of the contents of the alimentary tract.

The results of pH determinations of the contents of different parts of the alimentary tracts of 3 groups of 5 chickens that had been fed for 24 hr. on either mash, whole wheat or fine wheat meal are summarized in Table I. A significantly

TABLE I.—*Average pH of the Contents of the Alimentary Tract of Groups of 5 Chickens Fed Different Diets.*

Diet.	pH of contents of					
	Crop.	Gizzard.	Duodenum.	Ileum.	Caeca.	Rectum.
Mash	4·8	3·7	5·8	5·9	5·5	6·9
	(4·4–5·7)	(3·3–4·1)	(5·7–5·9)	(5·4–6·7)	(5·0–5·9)	(6·1–7·5)
Whole wheat	4·8	2·6	6·0	5·9	5·3	6·3
	(4·6–5·5)	(2·4–2·9)	(6·0–6·1)	(5·6–6·1)	(4·7–5·6)	(5·8–6·5)
Fine wheat Meal	4·4	2·5	5·9	6·1	5·8	6·3
	(4·1–4·6)	(2·3–2·9)	(5·8–6·2)	(5·9–6·3)	(5·4–6·0)	(5·8–6·5)

The range of pH values is shown in brackets.

higher pH was found in the gizzard contents of the chickens fed mash than in those fed whole wheat or fine wheat meal, an average pH of 3·7 compared with 2·6 and 2·5 respectively. No significant difference was noted between these 3 groups of chickens when the pH of the contents of the other organs were compared; no determinations were made of the proventriculus since this organ was usually empty. Similar results were obtained when chickens were maintained on these 3 foods for longer periods of time. For example, the pH of the gizzard contents of 6 chickens fed mash only for 7 days were 3·9, 3·8, 3·7, 3·4, 3·4 and 3·1. The corresponding figures for a group fed whole wheat only for 7 days were 2·9, 2·8, 2·8, 2·7, 2·6 and 2·6.

The results of pH determinations of gizzard contents of groups of 30 chickens fed either mash, whole wheat, whole wheat + 15 per cent fish meal, fine wheat meal, fine wheat meal + 15 per cent fish meal, fine wheat meal + 10 per cent casein, fine wheat meal + 20 per cent middlings, coarse wheat meal or coarse wheat meal + 15 per cent fish meal are summarized in Table II ; the chickens

TABLE II.—*pH of Gizzard Contents of Groups of 30 Chickens Fed Different Diets.*

Diet.	Average pH.	Range pH.
Mash	3·85	2·9–4·5
Whole wheat	2·75	2·4–3·3
„ „ + 15% fish meal	3·75	2·9–4·5
Fine wheat meal	2·74	2·3–3·3
„ „ „ + 15% fish meal	3·89	3·0–4·5
„ „ „ + 10% casein	2·73	2·4–3·2
„ „ „ + 20% middlings	2·75	2·3–3·2
Coarse wheat meal	2·78	2·3–3·6
„ „ „ + 15% fish meal	3·77	3·1–4·5
Nothing for 24 hr.	2·74	2·3–3·2

had been kept on these diets for periods of 4–24 hr. but since no relationship was noted between time and pH levels the results are considered together. One group of chickens that had not been fed after the preliminary fasting period

was included in this experiment. Very little difference was noted between the results for chickens fed whole wheat, coarse wheat meal or fine wheat meal, the average of the pH determinations being 2·75, 2·78 and 2·74 respectively. The addition of 20 per cent middlings to fine wheat meal had no effect on the pH of the gizzard contents, the average reading being 2·75. The addition of 15 per cent fish meal to whole wheat, coarse wheat meal, and fine wheat meal resulted in gizzard pH readings similar to those obtained when mash was fed, *i.e.*, an average of 3·75–3·89. Increased pH readings, however, were not observed when 10 per cent casein was added to fine wheat meal. Low pH values were obtained in chickens starved for 18 hr., the average pH of the resting gizzard being 2·74.

Some determinations were also made with fine wheat meal containing 10 per cent fish meal and high pH readings were again noted, these being only a little lower than when 15 per cent fish meal was added.

The effect of adding acid to different foods.

For this experiment 20 g. of different foods were mixed with 100 ml. of distilled water, the pH measured, and re-measured after adding N-HCl in increasing amounts. The results for mash and fine wheat meal with and without the addition of 15 per cent fish meal are shown in Table III. The most significant

TABLE III.—*Effect of Adding Normal Hydrochloric Acid to Different Foods.*

Total N-HCl added (ml.).	pH of suspensions* of F.W.M.	F.W.M. + 15% fish meal.	Mash.
0	5·8	6·1	5·9
1·0	4·4	5·0	5·1
2·0	3·6	4·6	4·7
3·0	2·9	4·2	4·2
4·0	2·6	3·8	3·9
5·0	2·3	3·5	3·6

F.W.M. = Fine wheat meal.

* 20 g. of each food were suspended in 100 ml. of distilled water.

are those following the addition of 3·0 and 4·0 ml. of acid since the pH of the fine wheat meal suspension was then 2·9 and 2·6 respectively, such readings being frequently found in the gizzard contents of chickens fed this diet. The corresponding readings for mash, 4·2 and 3·9, and for fine wheat meal + 15 per cent fish meal, 4·2 and 3·8, were also similar to those found in gizzard contents of chickens fed those diets.

The fate of Salm. gallinarum *in the alimentary tract following its administration in different foods.*

Estimations were made of the numbers of viable *Salm. gallinarum* in the contents of different parts of the alimentary tract of chickens, the food of which consisted either of mash, mash + 20 per cent alkali, whole wheat, whole wheat + 20 per cent alkali, whole wheat + 15 per cent fish meal, fine wheat meal, fine wheat meal + 20 per cent middlings, fine wheat meal + 15 per cent fish meal,

coarse wheat meal or coarse wheat meal + 15 per cent fish meal, into which had been mixed a known amount of *Salm. gallinarum*. Five chickens were used for each food and they were killed at 2, 4, 6, 8 and 24 hr. after the start of the experiment. Except for chickens killed at 2 hr. when *Salm. gallinarum* had not reached the rectum, the effect of time was unimportant. A much more important factor was the degree of acidity of the gizzard contents. Because of this the results shown in Table IV do not represent the average for the 5 chickens fed each food

TABLE IV.—*Fate of* Salm. gallinarum *in the Alimentary Tract of Chickens Following Administration in Different Foods.*

	Salm. gallinarum × 10^4/ml., in						
	Food.	Crop.	Gizzard.	Duodenum.	Ileum.	Rectum.	Gizzard pH.
Mash	200	250	45	75	26	55	3·6
„ + 20% alkali	500	650	180	120	80	70	5·2
Whole wheat	325	225	0	0	0	0	2·5
„ „ + 20% alkali	425	300	200	150	60	50	6·1
„ „ + 15% fish meal	550	300	45	35	30	27·5	3·9
Fine wheat meal	500	600	1·0	6·0	5·5	10·0	2·5
„ „ „ + 15% fish meal	550	600	65	150	110	100	4·0
„ „ „ + 20% middlings	400	500	7·5	3·5	3·5	1·5	2·7
Coarse wheat meal	350	600	0	3·0	4·5	3·0	2·8
„ „ „ + 15% fish meal	750	800	30	100	150	200	3·6

but a typical example of the results obtained with each. Complete destruction of *Salm. gallinarum* appeared to be occurring in the gizzard of the chicken fed whole wheat and a significant degree of destruction in the gizzards of the chickens fed coarse wheat meal, and fine wheat meal with and without the addition of 20 per cent middlings. Very much less destruction occurred in chickens fed diets containing fish meal or fed mash. It is probable that no destruction took place when *Salm. gallinarum* was administered in the diets containing alkali, the somewhat lower counts observed in the gizzard and in the organs posterior to it being accounted for by dilution with secretions of the alimentary tract itself. The counts of the gizzard contents were often much lower than those in the duodenum. This was attributed to the definite but short interval of time that elapsed between killing a chicken and removing its gizzard contents, during which additional bacterial destruction occurred. Apart from the gizzard action and its relation to pH, another significant finding was that little or no difference was observed between the counts of *Salm. gallinarum* in the contents of the duodenum, ileum, and rectum of the chickens used in this experiment indicating that no appreciable destruction of *Salm. gallinarum* occurred in these organs.

The fate of Salm. gallinarum *when added to the contents of different parts of the alimentary tract.*

The results of estimations of the numbers of viable *Salm. gallinarum* in gizzard contents of different pH at intervals of time after adding an 18-hr. broth culture, 0·02 ml. per ml. of contents, are shown in Table V. The gizzard contents were taken from a group of chickens immediately after death and were selected so as to give a suitable range of different pH values. They were placed in boiling tubes in a water-bath at 38–39°. The results show a direct relationship between the pH

TABLE V.—*Fate of* Salm. gallinarum *Added to Gizzard Contents of Different pH.*

Minutes after addition of *Salm. gallinarum.*	*Salm. gallinarum* × 10^4/ml., in gizzard contents of pH of								
	2·5	2·7	2·8	2·9	3·0	3·2	3·4	3·6	7·0*
0	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000
2½	60	125	200	350	225	520	525	1,200	900
5	0·85	65	60	260	275	660	600	650	850
10	0	0·55	8	425	155	350	225	500	850
20	0	0	0	0·6	5	26	125	300	400
30	0	0	0	0	0	35	70	450	750
40	0	0	0	0	0	0	25	300	500
180	—	—	—	—	—	—	—	—	500

* Gizzard contents, originally pH 2·5, neutralized with NaOH. Only one of the gizzard contents was counted at 0 min.

of the gizzard contents and the survival time of *Salm. gallinarum* ; bacteria were destroyed very rapidly at pH 2·5 and progressively more slowly as the pH increased until pH 3·6 when little or no destruction took place within 40 min. No destruction of *Salm. gallinarum* occurred within 180 min. in gizzard contents originally of pH 2·5 but adjusted to pH 7·0 with NaOH, indicating that the lethal effect in the gizzard was related to acidity. These experiments were repeated with different gizzard contents and 4 different cultures of *Salm. gallinarum* ; the results closely resembled those shown in Table V.

Similar experiments, using the contents of the crop, duodenum and ileum, but with a smaller inoculum of *Salm. gallinarum*, are illustrated in Table VI. In contrast to gizzard contents, no destruction of *Salm. gallinarum* occurred within 4 hr. of incubation. In fact, actual multiplication took place in the duodenal and ileum contents. A much smaller number of viable *Salm. gallinarum* was present in the crop contents at 6 hr. where the pH had dropped from an initial reading of 5·6 to 4·2, presumably due to the activity of other bacteria. When this pH drop was prevented in another experiment by the addition of alkali, the destruction did not occur, a slight degree of multiplication being noted instead. The experiments with duodenal and ileum contents were repeated with material from different chickens ; a degree of multiplication similar to that shown in Table VI always took place in duodenal contents. Occasionally, no multiplication occurred in ileum contents, a progressive decrease in the *Salm. gallinarum* count being noted at, and after, 4 hr. incubation. Experiments indicated that this was probably due to competitive effects of other bacteria not accompanied by a marked decrease in pH as was the case with crop contents.

TABLE VI.—*Fate of* Salm. gallinarum *Added to Crop, Duodenum and Ileum Contents.*

Hours after addition of *Salm. gallinarum.*	*Salm. gallinarum* × 10^4/ml., in contents of		
	Crop.	Duodenum.	Ileum.
0	75	60	110
1	50	50	125
2	120	75	125
4	75	600	650
6	1	3,500	1,200
8	0	2,750	1,000

The relationship between gizzard pH and the number of viable Salm. gallinarum *entering the duodenum of chickens fed different foods containing* Salm. gallinarum.

The results of pH determinations of gizzard contents made at the same time as estimations were carried out of the numbers of viable *Salm. gallinarum* in the material entering the duodenum of groups of 12 chickens which were being fed different foods containing approximately 10^7 *Salm. gallinarum* per g. are summarized in Table VII. These estimations were made immediately the chickens were

TABLE VII.—*Relationship between Gizzard pH and Number of* Salm. gallinarum *Entering the Duodenum of Groups of* 12 *Chickens Fed Different Foods Containing* Salm. gallinarum

Food.	Gizzard pH		Duodenal count $\times 10^4$/ml.	
	Average.	Range.	Average.	Range.
Mash	3·7	2·7–4·4	67·4	0·4–200·0
Whole wheat	2·7	2·5–2·9	0·1	0– 0·3
„ „ + 15% fish meal	3·7	2·9–4·3	48·0	1·0–150·0
Fine wheat meal	2·8	2·5–3·2	7·1	0·9– 20·0
„ „ „ + 20% middlings	2·7	2·3–3·2	8·4	0– 40·0
„ „ „ + 15% fish meal	3·8	3·3–4·5	83·8	25·0–225·0
Coarse wheat meal	2·7	2·4–3·1	3·2	0– 15·5
„ „ „ + 15% fish meal	3·7	3·1–4·8	70·8	4·0–155·0

Each food contained approximately 10^7 *Salm. gallinarum* per g.

killed. This was usually 4 hr. after they were allowed to begin eating the infected food which, as in previous experiments, was provided *ad lib.* for the whole 4 hr.

Duodenal counts were by far the lowest in chickens fed whole wheat ; no *Salm. gallinarum* were found in the duodenum of 7 of 12 chickens. Higher duodenal counts were noted in chickens fed either coarse wheat meal, fine wheat meal or fine wheat meal + 20 per cent middlings despite the fact that the gizzard pH readings were very much the same in all 4 groups of chickens. The addition of 15 per cent fish meal to the 3 forms of wheat resulted in much higher pH readings of the gizzard contents and in much higher numbers of *Salm. gallinarum* entering the duodenum, the results resembling those for mash. Any difference existing between whole wheat + fish meal and fine wheat meal + fish meal was very slight compared with the difference between whole wheat and fine wheat meal.

When allowances were made for the discrepancies expected in an experiment of this nature, a direct relationship was noted, as far as each food was concerned, between the pH of the gizzard and the number of *Salm. gallinarum* entering the duodenum. For example, the pH of the gizzard of the 12 chickens fed mash was 2·7, 2·9, 3·4, 3·5, 3·6, 3·8, 3·9, 3·9, 4·0, 4·1, 4·2, and 4·4 and the corresponding duodenal counts, $\times 10^4$/ml., were 0·8, 0·4, 22, 20, 40, 60, 25, 40, 100, 100, 200 and 200 respectively.

Mash and wheat as vehicles of Salm. gallinarum *infection ; the effect of the addition of alkali.*

The infection rate that occurred when 50×10^5 *Salm. gallinarum* mixed in mash or whole wheat were fed to chickens is shown in Table VIII. Of a total of 92 chickens given *Salm. gallinarum* in mash, 63 became infected, 31 of which died.

TABLE VIII.—*Effect of Giving Chickens* 50 × 10^5 Salm. gallinarum *in Mash or in Whole Wheat.*

Number of chickens in each group.	Number infected when *Salm. gallinarum* was given in	
	Mash.	Whole wheat.
23	15 (9)	1 (1)
15	11 (7)	4 (2)
18	13 (5)	2 (1)
36	24 (10)	4 (0)
Total 92	63 (31)	11 (4)

The figures in brackets represent the number of infected chickens that died.

When the same dose of *Salm. gallinarum* mixed with whole wheat was fed to the same number of chickens only 11 became infected, 4 of which died.

The results of administering different doses of *Salm. gallinarum* in mash or whole wheat, both with or without the addition of 20 per cent alkali, to groups of 23 chickens is illustrated in Table IX. In the mash groups the numbers of

TABLE IX.—*Effect of Giving Chickens Different Doses of* Salm. gallinarum *in Mash or Whole Wheat, With and Without the Addition of Alkali.*

Vehicle.	Number of 23 chickens infected when the number of *Salm. gallinarum* bacteria, × 10^5, given was		
	500	50	5
Mash	20 (12)	15 (9)	7 (1)
„ + 20% alkali	23 (17)	21 (10)	18 (7)
Whole wheat	11 (6)	1 (1)	1 (0)
„ „ + 20% alkali	—	22 (5)	12 (3)

The figures in brackets represent the number of infected chickens that died.

chickens that became infected after receiving doses of 500 × 10^5, 50 × 10^5 and 5 × 10^5 viable *Salm. gallinarum* were 20, 15 and 7 respectively. The corresponding figures for mash containing 20 per cent alkali were 23, 21 and 18 and for whole wheat 11, 1 and 1. Experiments at the highest dose level were not carried out with wheat containing 20 per cent alkali; the figures for the two lower dose levels were 22 and 12.

The effect of feeding chickens different amounts of Salm. gallinarum *in whole wheat and wheat meals with and without the addition of fish meal or middlings.*

The numbers in groups of 36 chickens that became infected after consuming different amounts of *Salm. gallinarum* contained in either whole wheat, coarse and fine wheat meals, these 3 materials containing 15 per cent fish meal, or in fine wheat meal containing 20 per cent middlings are shown in Table X. The numbers of chickens that became infected after being given 50 × 10^5 viable *Salm. gallinarum* in whole wheat, coarse wheat meal and fine wheat meal were 4, 11 and 18 respectively. When to these foods 15 per cent fish meal was added, the corresponding figures were 29, 29, and 30. This increase in the infection rate was not noted with fine wheat meal containing 20 per cent middlings, 19 chickens becoming infected.

TABLE X.—*Effect of Feeding Chickens Different Amounts of* Salm. gallinarum *in Whole Wheat and Wheat Meals With and Without the Addition of Fish Meal or Middlings.*

Vehicle.	Number of 36 chickens infected when the number of *Salm. gallinarum* bacteria, $\times 10^5$, administered was		
	50	15	5
Whole wheat	4 (0)	—	1 (0)
,, ,, + 15% fish meal	29 (12)	—	12 (7)
Coarse wheat meal	11 (2)	—	—
,, ,, ,, + 15% fish meal	29 (13)	—	14 (8)
Fine wheat meal	18 (9)	9 (6)	4 (2)
,, ,, ,, + 15% fish meal	30 (14)	24 (12)	12 (5)
,, ,, ,, + 20% middlings	19 (8)	—	—
1 ml. distilled water after fasting	14 (7)	—	—

The figures in brackets represent the number of infected chickens that died.

When the same dose was given in 1 ml. of distilled water to 36 chickens that had been fasted for 18 hr., 14 became infected.

When 15×10^5 *Salm. gallinarum* in fine wheat meal containing 15 per cent fish meal were fed to 36 chickens, 24 became infected; but only 9 of 36 with fine wheat meal.

When the dose level was reduced to 5×10^5 viable *Salm. gallinarum* a similar relationship was noted between the results obtained with different foods. The numbers of chickens that became infected in the whole wheat + fish meal, coarse wheat meal + fish meal and fine wheat meal + fish meal groups were closely similar, 12, 14 and 12 respectively. Only one chicken became infected in the whole wheat group and 4 in the fine wheat meal group. As in most of the experiments about half the infected birds died.

The effect of feeding whole wheat or mash on the spread of Salm gallinarum *infection.*

For this experiment 2 groups of 50 chickens were housed in 2 identical outdoor arks on the ground. An additional 20 chickens were infected by giving each of them 500×10^5 *Salm. gallinarum* in alkali. After an interval of 2 hr. 10 of these chickens were placed in one of the arks and 10 in the other. The inhabitants of one ark were then fed on mash only and the inhabitants of the other on whole wheat only; the diet was not varied for the duration of the experiment, neither were the arks moved. Dead chickens were removed as soon as they were found. At weekly intervals after the first 3 weeks all the in-contact chickens were tested for agglutinins to *Salm. gallinarum* by the rapid whole-blood test; chickens giving positive reactions were recorded as being infected but were not removed. Table XI shows that the total number of in-contact chickens that were classed as infected at 3 weeks was 12 in the mash-fed group and 3 in the wheat-fed group. At 4 weeks the corresponding figures were 21 and 6, at 5 weeks 21 and 6, and at 6 weeks 23 and 7. No evidence of further spread of infection was noted at subsequent tests and examination of all chickens at the 7th week revealed that none of them were excreting *Salm. gallinarum* in their faeces. At the end of the experiment all the surviving birds were killed. Lesions characteristic of *Salm. gallinarum* infection were noted in all the chickens that had been classified as infected but in none of the remainder.

TABLE XI.—*Effect of Feeding Whole Wheat or Mash on the Spread of* Salm. gallinarum *Infection.*

Time, in weeks, after introducing 10 infected chickens.	Total number of 50 in-contact chickens that became infected.	
	Wheat-fed.	Mash-fed.
3 . .	3 (1)	12 (3)
4 . .	6 (2)	21 (5)
5 . .	6 (4)	21 (6)
6 . .	7 (4)	23 (8)

The figures in brackets represent the number of infected chickens that died.

The above experiment was repeated but the results were nullified because a number of chickens dying in the whole wheat group were eaten by other members of the group, a phenomenon not uncommon in chickens fed wheat only.

DISCUSSION.

These results show that the diet of chickens at the time they are exposed to infection with bacteria whose portal of entry is the intestinal tract may be an important factor in determining whether or not infection will take place. It is apparent that the reason for the different infection rates that were obtained when *Salm. gallinarum* was administered in different foods was that these foods did not influence the bactericidal action of the gastric juice to the same extent. At least two properties of foodstuffs appear to influence this bactericidal mechanism, their capacity to neutralize gastric acidity and their physical consistency. The first was well illustrated by the experiments with foods containing fish meal. This substance was responsible for a much higher pH in gizzard contents, and consequently, a lesser degree of destruction of *Salm. gallinarum*, a greater number of these bacteria entering the duodenum, and a higher infection rate. The effect of the consistency of food was brought out by comparing the results obtained with whole wheat with those obtained with wheat meals. Despite the fact that similar pH readings of the gizzard contents were found in chickens fed either of these diets, a greater degree of destruction of *Salm. gallinarum* occurred in the gizzard of those fed whole wheat than in those fed wheat meals, and a much lower infection rate in the former than in the latter. The differences are, undoubtedly, due to the fact that *Salm. gallinarum* is exposed to the action of gastric juice for a much longer time with a consequent greater degree of bacterial destruction in chickens fed whole wheat, since this food is retained in the gizzard until it is ground to a consistency like that of fine wheat meal before it is released into the duodenum. It is noteworthy that this time factor was of little or no importance when diets of different consistency, but containing fish meal, were compared. The average pH of the gizzard contents of chickens fed such diets, however, was approximately 3·8 compared with approximately 2·7 when whole wheat or wheat meals were fed. The *in vitro* studies using gizzard contents of different pH indicated that destruction of *Salm. gallinarum* was rapid at pH 2·7 but sufficiently slow at pH 3·8 to be uninfluenced by the different times that foods of different physical consistency would be retained in the gizzard undergoing digestion.

In the early part of this work it was considered likely that protein, particularly fish meal, was responsible for the high pH readings obtained in the gizzard contents of chickens fed mash, and the experiments were planned to determine whether or not this was so. Although the fish meal was proved to be responsible, the experiments in which chickens were fed fine wheat meal + 10 per cent casein, a diet containing a similar amount of protein to that in a diet of fine wheat meal + 15 per cent fish meal, showed that the pH of the gizzard contents of those chickens was no higher than that obtained when fine wheat meal alone was fed. It is therefore much more likely that the high ash content, and not the protein, of fish meal was responsible for the high pH obtained in the gizzard of chickens fed diets containing this substance, particularly as the ash, derived mainly from fish bones, would be basic in character.

The fact that *Salm. gallinarum* infection spread more rapidly from infected to healthy chickens fed on mash than to healthy chickens fed on whole wheat indicates the practical importance of diet in the induction of *Salm. gallinarum* infection in chickens, particularly as mash and whole wheat form the staple food of most chickens. It is probable that when a natural outbreak is diagnosed in a flock it would be worth while to alter the diet to whole wheat only, until it is possible to institute other methods of control. Under certain conditions, it is conceivable that this principle of altering the diet may be applicable in outbreaks of disease in animals other than chickens.

In many investigations on the relationship between the nutritional level of the host and resistance to diseases such as salmonella infection, the experimental disease has been produced by the oral administration of a known number of bacteria to groups of animals fed on different diets. It is obvious from the present work that such a procedure is open to criticism on the ground that more bacteria may be destroyed by the gastric mechanism in the animals fed one diet than in those fed another. As a consequence, incorrect conclusions might be drawn from the results obtained. This has also been pointed out by Arnold (1927*b*). To overcome such variations it might be advisable in nutrition experiments to infect animals by withholding food from them for 12–18 hours and then to administer the infective dose in an alkaline vehicle by mouth.

SUMMARY.

Different infection rates were observed when different foods containing similar numbers of *Salm. gallinarum* were fed to chickens.

The main reason for this was that certain properties of some of the foods had a profound influence on the bactericidal action of the gastric juice in the gizzard.

One of these properties was physical consistency and another was the ability to maintain a relatively high pH in the gizzard.

I am grateful to Mr. W. E. Crabb, A.I.M.L.T. for his capable technical help, and to Dr. R. F. Gordon of the Animal Health Trust's Poultry Laboratory for providing the facilities for carrying out this work. Help or advice has also been received at different stages of the work from Messrs. A. N. Worden and L.G. Chubb and Miss Gillian Anderson. Finally, I wish to express my grateful thanks to Mr. F. Tabbitt and his staff for the careful manner in which they have looked after the large number of birds used in the study.

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CHOLINESTERASE INHIBITION AT THE MYONEURAL JUNCTION.

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WHEN a rat or a rabbit is given a lethal dose of some anticholinesterase drugs of the organo-phosphorus group, it can often be resuscitated by artificial respiration. Since artificial respiration is a recognised part of therapy in human cases of poisoning it is important to know why artificial respiration succeeds in animals after some compounds and fails after others. In these animals atropine has been given to overcome the muscarinic effects and these together with central effects (Koelle and Gilman, 1949; Douglas and Matthews, 1952) will not be discussed in this paper which is concerned with events at the myoneural junctions in the rat diaphragm.

The older view that the organo-phosphorus compounds produce irreversible inhibition of cholinesterase is gradually giving way to the idea that the enzyme-inhibitor complex is not completely stable and that a small amount of reversal of inhibition occurs, at least with some of the organo-phosphorus compounds. The success of artificial respiration might depend on keeping the animal alive until sufficient cholinesterase has reappeared by breakdown of the enzyme-inhibitor complex to allow the diaphragm and the other respiratory muscles to function. This would explain the success of artificial respiration in rats poisoned with di-ethyl *p*-nitrophenyl phosphate (paraoxon) and tetraethyl pyrophosphate (TEPP), and the failure after di-isopropyl phosphoro-fluoridate (DFP) could be accounted for by attributing greater stability to the DFP-enzyme complex. This hypothesis has been examined by determining the levels of cholinesterase in the diaphragm and at the myoneural junctions after a lethal dose and after resuscitation, and by assessing the degree of reversal of inhibition at the myoneural junctions after poisoning with paraoxon, TEPP and DFP.

The interpretation of these findings is complicated by the presence of two cholinesterases, the so-called specific and non-specific, in the diaphragm. An attempt has been made to determine the contribution of each enzyme to recovery by examining their distribution in the diaphragm and by following their relative activities at varying stages during the process of recovery.

METHODS.

Histochemical.

The Koelle (1950) method for cholinesterase was used with the omission of the step involving saturation of the substrate with copper thiocholine (Reagent 8 in Koelle's method).

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The acetyl thiocholine was made in this laboratory by Mr. A. N. Davison by the method of Renshaw, Dreisbach, Ziff and Green (1938). The butyryl thiocholine was a gift from Roche Products Ltd.

Inhibitors.—Many of the organo-phosphorus compounds have been renamed, and the new names are given here, but long-recognized abbreviations such as DFP are retained. The following inhibitors were used :

Paraoxon	.	diethyl *p*-nitrophenyl phosphate.
TEPP	.	tetraethyl pyrophosphate.
DFP .	.	di-isopropyl phosphorofluoridate.
Mipafox	.	di-isopropyl phosphorodiamidic fluoride.
NU.1250	.	the N-*p*-chlorophenyl-N-methyl carbonate of *m*-hydroxy-phenyl trimethyl ammonium bromide.
284.c.51	.	1–5-bis (allyl dimethyl ammonium phenyl) pentane-3-ene dibromide.

Animals.

These were all albino rats. Some were killed with coal gas and others by the subcutaneous injection of a lethal dose of paraoxon, TEPP or DFP.

For the experiments dealing with the recovery of cholinesterase activity *in vivo*, uniform severity of symptoms was produced in the groups of poisoned animals by injecting half the LD_{50} dose followed every 20 min. with $\frac{1}{10}$ of the LD_{50} dose until each individual rat was showing severe symptoms. This was done with paraoxon, TEPP and DFP. It was assumed that rats showing equal symptoms had their cholinesterase equally inhibited. Certainly this method gave more reproducible results than the administration of a single dose to rats.

Rat Diaphragms.

Frozen sections.—These were cut at 10–15μ from unfixed diaphragms of both normal and poisoned animals. The piece of diaphragm used for sectioning was selected so that each section would contain part of the phrenic nerve and hence a line of myoneural junctions. This technique has been described more fully elsewhere (Denz, 1953).

Treatment of sections with inhibitors.—To study the reversal of cholinesterase inhibition *in vitro* sections were placed for 30 min. at 37° in aqueous solutions of inhibitors. For complete inhibition the concentrations were : paraoxon (10^{-5} M), TEPP (10^{-5} M), DFP (10^{-4} M). The sections were removed from the inhibitors and washed three times in 0·9 per cent saline. The sections were then placed in saline in the dark at room temperature (17–21°). Other sections were kept at 4° in the cold room and at 37° in an incubator. Sections were taken at varying times up to 6 days for histochemical examination.

Treatment of whole diaphragms with inhibitors.—The diaphragms were dissected out in one piece, washed free from blood under the cold water tap, then treated with aqueous solutions of the inhibitors at 37° for 30 min. They were then washed six times in 0·9 per cent saline. Some were taken immediately for cholinesterase estimations, others were kept in saline at 37° for up to 13 days.

Warburg Determinations.

The technical details described by Aldridge (1950) were followed. Acetylcholine was used at a final concentration of 0·015 M and acetyl-β-methylcholine at 0·03 M. Estimations were done at a pH of 7·6.

The activity of the thiocholine esters was measured at pH 6·0, because this pH is used in the histochemical method. The buffer of pH 6·0 was obtained by using 0·012 M sodium bicarbonate and equilibrating wth 40 per cent CO_2–60 per cent N_2 (Koelle, 1950). The final substrate concentration of acetyl thiocholine was 0·005 M.

Rat diaphragm, brain, heart and harderian gland were homogenized in bicarbonate buffer and aliquots were taken for cholinesterase determinations. Horse serum was sterilized by Seitz filtration and was used without further treatment. All results were corrected for non-enzymic hydrolysis and for acid production or CO_2 absorption in the absence of substrate.

To determine the effect of paraoxon on specific cholinesterase as shown in Fig. 2 and to measure in particular levels of activity below 10 per cent of normal, one preparation of rat brain was made. Ten rat brains were homogenized, made up to a volume of 80 ml. in

bicarbonate buffer and stored in 10 ml. lots in the frozen state at – 4°. The stock solution of 0·21 M acetyl-β-methylcholine and the serial dilutions of paraoxon were also stored in the frozen condition. For use, preparations were warmed to 37° and to 1 ml. of the rat brain suspension was added 4 ml. of bicarbonate buffer and 1 ml. of the appropriate dilution of the inhibitor. This mixture was held at 37° for 30 min. and then the reaction was stopped by adding 1 ml. of 0·21 M acetyl-β-methylcholine. Aliquots of 0·5–6 ml. depending on the residual activity of the enzyme were taken into Warburg flasks and diluted to a final volume of 6 ml. with bicarbonate buffer containing 0·03 M acetyl-β-methylcholine.

The separate contributions of specific and non-specific cholinesterase to the hydrolysis of acetylcholine by the rat diaphragm have been determined by the use of selective inhibitors. In each experiment two diaphragms were homogenized in bicarbonate buffer to a final volume of 10 ml. A 3 ml. aliquot was taken for total cholinesterase activity as measured by the hydrolysis of acetylcholine. A second aliquot of 3 ml. was taken into a Warburg flask and mipafox was added to give a final concentration of 1×10^{-7} M and the mixture was held at 37° for 30 min.; acetylcholine was then added to give a final concentration of 0·015 M, and the residual activity determined. Under these conditions mipafox selectively inhibits all the non-specific cholinesterase but leaves the specific cholinesterase unaffected. A third aliquot of 3 ml. was taken into a Warburg flask and either NU.1250 at a final concentration of 10^{-6} M or 284.c.51 at a final concentration of 3×10^{-4} M, and acetylcholine added to give a final concentration of 0·015 M. Here specific cholinesterase is completely inhibited and non-specific cholinesterase unaffected. The data in Table V were obtained in this way and the sum of the activities of the two enzymes determined separately was very close for the value for the total activity of the cholinesterase in the diaphragm. In Table IV and Fig. 5 the total activity has been determined together with the activity of one of the two enzymes and the activity of the second enzyme has been obtained by difference.

Expression of Results.

Groups of figures have been expressed as means and standard errors in the Warburg units as micromols. of CO_2 evolved per min. per g. of tissue. Many of the figures for activity of cholinesterase are expressed as a percentage of the activity of the normal diaphragm. The difficulty arises that there is considerable variation in the cholinesterase activity of the normal diaphragm. Thus with 25 diaphragms giving a mean and standard error of 28·33 ±0·96 μl CO_2/min./g. of diaphragm there is a range of 13·47–32·64. Much of this variation is due to the fact that small diaphragms have more cholinesterase activity per g. than larger. In Fig. 1 the relation of weight of diaphragm to cholinesterase activity per g. is shown.

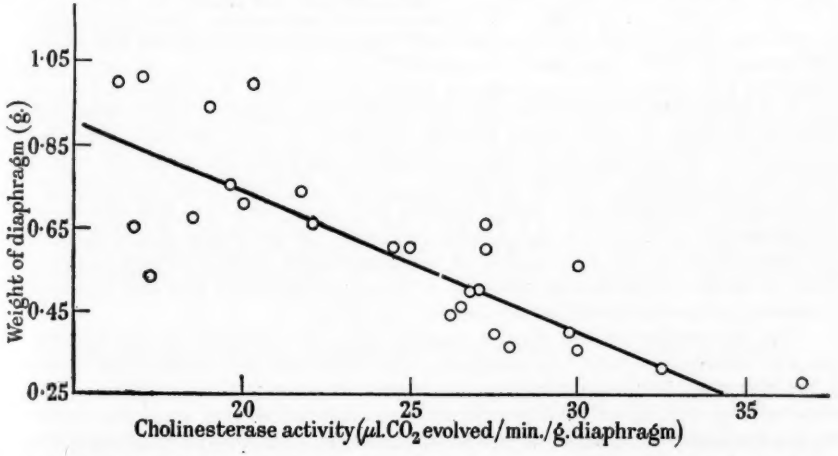

FIG. 1.—Relation of weight of diaphragm to cholinesterase activity (substrate-acetylcholine).

A line has been fitted to these points and will give the expected normal value for any given weight of diaphragm, and this value can be used as the divisor in calculating the per cent activity of cholinesterase in the diaphragm from a poisoned animal.

RESULTS.

Standardization of the Koelle Method.

When the Koelle histochemical method for cholinesterase is applied to frozen sections of fresh unfixed diaphragm each section shows a line of myoneural junctions that can be used as a means of demonstrating cholinesterase activity. The identification of the enzyme as cholinesterase is dealt with in a previous paper (Denz, 1953).

If frozen sections of a normal diaphragm are treated at 37° for 30 min. with an appropriate concentration of inhibitor (say 10^{-6}M paraoxon), staining of the sections is inhibited. By using lower concentrations of inhibitor (*i.e.*, $2–5 \times 10^{-8}$M paraoxon) partial inhibition of cholinesterase can be produced so that the depth of staining is reduced but the myoneural junctions can still be seen. The intensity of staining can be used as a rough indication of the amount of residual cholinesterase activity. In Table I the intensity of staining is compared with the residual cholinesterase activity.

TABLE I.—*Comparison of Intensity of Staining with Residual Cholinesterase Activity. Koelle's Cholinesterase Method with Rat Diaphragm.*

Intensity of staining.	Residual activity (per cent).	Description of staining.
±	<10	Barely perceptible brown smudge on muscle fibres, microscopic.
±	10–20	Clearly visible under microscope. Well defined end-plates, light brown.
+	30–50	Just visible to naked eye. Some internal structure; now dark brown.
++	60	Easily visible to naked eye. Dark blobs under microscope, fairly wide dispersion into sarcoplasm.

It is important to determine the significance of a negative result using Koelle's method because in some cases animals survived a toxic dose of paraoxon and yet had no cholinesterase at the myoneural junctions as shown by the histochemical method as usually employed. Again, isolated diaphragms that were functioning normally after being washed free from paraoxon did not show any cholinesterase activity by the Koelle method. On the basis of the necessity of cholinesterase for the functioning of the diaphragm, these findings suggest that there is some cholinesterase present in these preparations but the Koelle histochemical method is insufficiently sensitive to detect it.

The sensitivity of the Koelle method can be increased by prolonging the time of incubation with the substrate, and when this was raised from 30 min. to 4 hr. many of the previously negative results became positive. When paraoxon was used *in vitro* as an inhibitor for 30 min. and the section was then repeatedly washed in saline, incubation with the substrate for 4 hr. always resulted in staining of the myoneural junctions even when the concentration of paraoxon was as

high as 10^{-4} M (*i.e.*, 1000 times the concentration theoretically necessary to inhibit all the cholinesterase). In the diaphragms of rats killed with paraoxon staining always developed on incubation in the substrate for 3–4 hr., and the same can be said of isolated diaphragms that had been inhibited with paraoxon and then washed in saline until function had been restored. From Table I it can be seen that if the residual cholinesterase activity was less than 10 per cent of normal the unmodified Koelle technique would probably return this as negative. Lower levels than 10 per cent can be detected if the sections are left in the substrate for some hours.

An attempt was made to determine more precisely the sensitivity of the Koelle method by determining the intensity of staining after different periods of incubation in the substrate in relation to the degree of inhibition of the cholinesterase. Kinetic data (quoted below) for the inhibition of cholinesterase by paraoxon can be used to calculate the concentration of paraoxon necessary to produce known degrees of inhibition of cholinesterase in frozen sections of diaphragm. The use of these kinetic data is given more critical consideration later. The intensity of staining produced after varying times in the substrate was determined for each level of cholinesterase activity in the sections. Some of these results are given in Table II.

TABLE II.—*Relation of Intensity of Staining to Percentage Residual Activity of Cholinesterase. Koelle Histochemical Method. Inhibitor: Paraoxon.*

Incubation time (in substrate).	Residual activity as percentage.									
	0·01	0·1	1	5	6	10	15	20	30	100
10 min.	−	−	−	−	−	−	−	−	±	±
20 „	−	−	−	−	−	−	±	±	±	+
30 „	−	−	−	−	±	±	±	±	+	++
1 hr.	±	±	±	±	±	±	±	+	++	++
2 „	±	±	±	±	+	+	++	++	..	..
3 „	+	+	+	++	++	++	..	..	..	..
4 „	++	++	++	++	..	..	..	..	..	..

From this it will be seen that any residual activity less than 5 per cent of original activity produces the same pattern of staining. This suggests that under these conditions 5 per cent of the cholinesterase is either not inhibited, whatever the concentration of paraoxon to which it has been exposed, or that 5 per cent of the cholinesterase inhibition is reversed under these conditions. That this activity is due to enzyme action is shown by the finding that the addition of paraoxon to the substrate at a concentration of 10^{-6} M completely inhibits staining however long the sections are incubated in the substrate. It is only when the sections have been carefully washed free from excess of inhibitor before being put into the substrate that a residual activity of 5 per cent can be demonstrated.

Levels of Cholinesterase Activity in Diaphragms of Poisoned Animals.

The conclusion that about 5 per cent of the original cholinesterase activity in the rat diaphragm reappears within a few hours after inhibition by paraoxon whatever the concentration above that necessary theoretically to produce 95 per cent inhibition (*i.e.*, 9×10^{-8} M) is supported by the fact that biochemical estima-

tions on the diaphragms of rats killed by paraoxon show some cholinesterase activity even though the dose was 100 times the LD_{50}. The mean and standard error was $1{\cdot}49 \pm 0{\cdot}52$ as compared with $28{\cdot}33 \pm 0{\cdot}96$ for normal diaphragms and indicates the presence of about 5 per cent of the original activity. Rats after resuscitation or just surviving a near-lethal dose of paraoxon gave a figure of $4{\cdot}47 \pm 0{\cdot}63$, *i.e.*, about 15 per cent of normal activity.

In rats killed with DFP usually no cholinesterase activity could be detected and the highest value obtained was 1·04. In rats just surviving a dose of DFP the value was $3{\cdot}43 \pm 0{\cdot}71$.

These findings have been confirmed by the histochemical method and are supported by the observations of Barnes and Duff (1953) that rat phrenic nerve-diaphragm preparations showed a considerable alteration in behaviour *in vitro* when the cholinesterase was reduced below 10 per cent.

Inhibition of Specific Cholinesterase by Paraoxon In Vitro.

From the above observations it is apparent that the main interest lies in the last 10 to 15 per cent of cholinesterase activity in the incompletely inhibited diaphragm, because it is at this level that functional failure or recovery occurs. The kinetic data used for assessing the percentage inhibition were derived from measurements of activity above 10 per cent of normal and there are objections to extrapolating beyond the limits of measurements as has been done in Table II. Data for levels below 10 per cent of normal activity were obtained by inhibiting a sufficiently active preparation of rat brain cholinesterase by paraoxon to give information at these low levels. The bimolecular rate constant calculated from data above the 10 per cent level in Fig. 2 is $1{\cdot}2 \times 10^6$ (min.$^{-1}$ l. mol.$^{-1}$) compared with the value $1{\cdot}1 \times 10^6$ recorded by Aldridge and Davison (1952) for sheep erythrocyte cholinesterase.

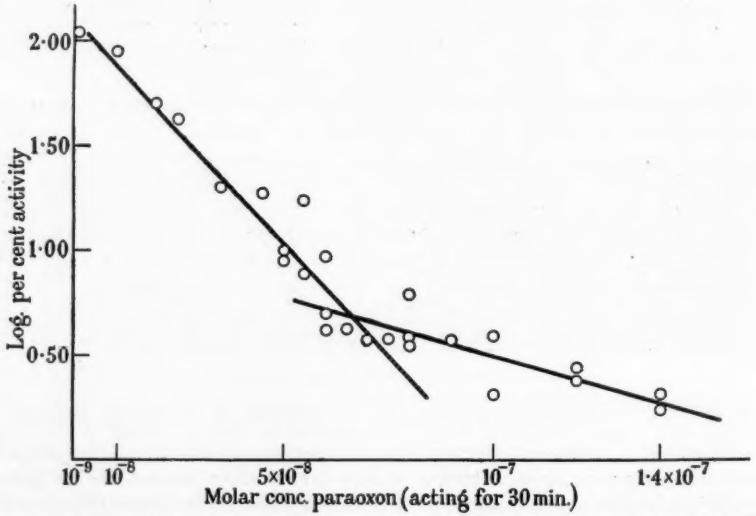

FIG. 2.—Inhibition of rat brain cholinesterase by paraoxon (substrate-acetyl-β-methycholine).

The significant finding in Fig. 2 is the change in slope below the 10 per cent level, where the inhibition actually produced is less than would be predicted from the kinetic data derived in the usual way from the upper part of the curve. Here again is evidence that the last trace of cholinesterase activity is relatively persistent when the activity is measured by the usual methods.

At this point it may be well to anticipate the next sections to some extent and point out that the estimation of cholinesterase activity by the histochemical and Warburg methods takes at least two hours and this is sufficient time for appreciable reversal of inhibition to take place. Any activity that is measured is the sum of the activity in the tissue at the time it was taken for estimation and the activity regenerated by reversal of inhibition that takes place during the estimation of the enzyme activity. At high levels of activity this factor of reversal during estimations is likely to be insignificant, but at levels of activity below 10 per cent of normal it may contribute considerably to the cholinesterase activity that is actually measured. This comment should not obscure the fact that 100 per cent inhibition is not demonstrated *in vivo* or *in vitro* with paraoxon unless an excess of inhibitor is present in the tissues up to the conclusion of the estimation of the cholinesterase activity.

Rate of Reversal of Inhibition In Vitro.

One of the factors contributing to the recovery of cholinesterase activity in the diaphragm may be the reversal of the combination between cholinesterase and the inhibitor. This was studied *in vitro* by treating sections of diaphragm with inhibitor, washing the sections free of inhibitor and then storing the sections at room temperature (17–21°). After treatment with high concentrations of paraoxon (10^{-5} M) and TEPP (10^{-5} M) sections had recovered 15 to 20 per cent of their cholinesterase activity in 24 hr., 30 to 40 per cent in 4 days and thereafter showed no further increase (Fig. 3). Recovery after paraoxon and TEPP was identical. On the other hand sections treated with DFP (10^{-4} M) behaved very differently and little or no reversal could be detected.

These experiments were carried out at room temperature because control sections kept at 37° soon lost their staining properties. However increase in temperature appears to cause a considerable increase in the rate of reversal of inhibition, as shown in Table III. An attempt was made to study reversal at 37° by following the cholinesterase activity in whole diaphragms removed from rats killed by paraoxon, but the cholinesterase levels of normal diaphragms kept at 37° fell rapidly and no accurate measure of reversal of inhibition was possible by this method.

TABLE III.—*Effect of Temperature on Reversal of Inhibition of Cholinesterase in the Diaphragm after Paraoxon. Activity Determined by Koelle Histochemical Method.*

Temperature.	Per cent recovery after: 1 day.	2 days.	3 days.
4°	5	6–15	15–20
17–21°	15–20	20–30	30–40
37°	30–40	—	—

The histochemical findings are offered as evidence of reversal of inhibition of the cholinesterase at the myoneural junctions after some inhibitors. No great precision of measurement is claimed. Although control tissues were examined for progressive inactivation as much as 30 per cent of the total activity could have been lost without detection by this method. The exponential form of the recovery curve may therefore be an artefact. On the other hand a study of reversibility *in vitro* may overestimate reversal *in vivo*, by assuming that the inhibited enzyme is not disposed of by some route other than reversal and that *in vivo* it remains at the myoneural junction for several days being removed only by breakdown of the enzyme-inhibitor complex with the liberation of active enzyme.

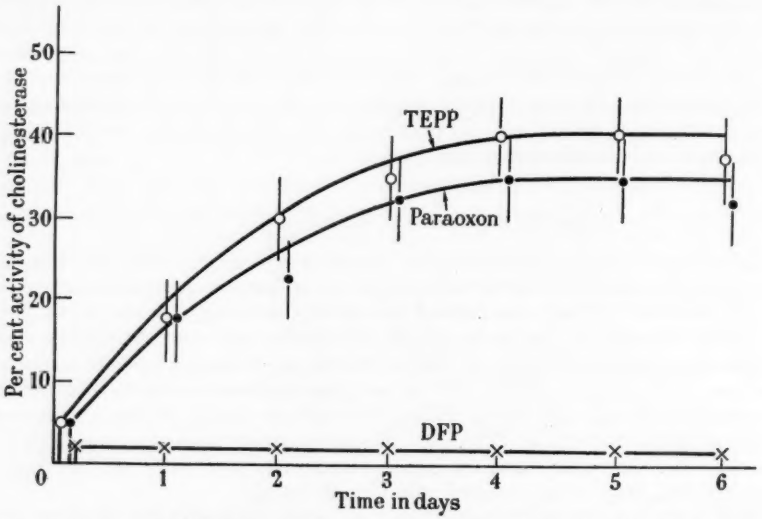

FIG. 3.—Recovery of cholinesterase activity at the myoneural junctions *in vitro* at room temperature (16–21°). Activity determined histochemically, vertical lines showing the range within which the results fall.

The Presence of Non-specific Cholinesterase in the Diaphragm.

The presence of the two cholinesterases in the diaphragm of the rat (Davison, 1953; Denz, 1953) leads to the necessity to study their individual contributions to the recovery of the total cholinesterase after poisoning. Fortunately it is possible to separate the effects of the two enzymes by using selective inhibitors, as described in the section on methods.

Relative activities of specific and non-specific cholinesterase in the whole diaphragm.—If the ability of these enzymes to hydrolyse acetylcholine is taken as the basis for comparison, the non-specific cholinesterase is about half as active as the specific cholinesterase in the whole diaphragm (Table IV).

Relative activities of the two enzymes at the myoneural junctions.—It has been generally accepted that the specific cholinesterase is localised at the myoneural

TABLE IV.—*Per Cent Hydrolysis of Acetylcholine Attributable to Specific and to Non-specific Cholinesterase in the Whole Diaphragm of the Normal Rat.*

Inhibitor used to separate effects of the two enzymes.	Number of estimations.	Mean per cent activity of:	
		Specific.	Non-specific.
Mipafox	8	71·0	29·0
284.c.51	4	64·9	35·1
NU.1250	6	63·9	36·1

junction. Some focal concentration of non-specific cholinesterase at the myoneural junction has been demonstrated by a histochemical method (Denz, 1953), but on the basis of the slowness of hydrolysis of butyryl thiocholine at the myoneural junctions it appeared likely that the non-specific cholinesterase possessed only a fraction of the activity of specific cholinesterase and certainly less than the 50 per cent suggested by the figures for the activity in the whole diaphragm given in Table IV.

This point was reinvestigated by studying histochemically the hydrolysis of acetyl thiocholine at the myoneural junctions when all the specific cholinesterase was inhibited by either 10^{-6} M NU.1250 or 3×10^{-4} M 284.c.51. These conditions permit the uninhibited action of non-specific cholinesterase. It is shown later in Table VI that the acetyl thiocholine used in this experiment was readily hydrolysed by non-specific cholinesterase. Under these conditions very little staining of the myoneural junctions was evident at 4 hr., and 8 or more hours' incubation was necessary to show the junctions clearly. Thus the activity at the myoneural junctions attributable to non-specific cholinesterase was less than 10 per cent of the total hydrolysis of acetyl thiocholine under these conditions.

This suggests that the non-specific cholinesterase activity may be distributed more widely throughout the muscle. The strictly defined distribution of the phrenic nerves and of the myoneural junctions in the diaphragm makes it possible to separate the neural from the aneural part with a pair of scissors. Histochemical examination of the two parts showed that the separation was reasonably precise. However, Table V shows that the aneural part contains rather more than half the specific cholinesterase activity found in the neural part, and this is more

TABLE V.—*Relative Activities of Specific and Non-specific Cholinesterase in Neural and Aneural Parts of Diaphragm Expressed as Percentage of Total Activity in the Neural Part.*

	Total cholinesterase.	Specific cholinesterase.	Non-specific cholinesterase.
Neural part	100	76·0	26·4
Aneural part	83·6	44·6	41·4

Each figure is the mean of four estimations of acetylcholine hydrolysis.

activity than might be anticipated for the aneural part. The non-specific cholinesterase showed a relative increase in the aneural part of the diaphragm. These findings support the view that specific cholinesterase is distributed chiefly at the myoneural junctions and that non-specific cholinesterase is present throughout the diaphragm, but it also suggests that some specific cholinesterase activity may be more widely distributed throughout the muscle. These comments on

the local distribution of enzymes in the diaphragm indicate the difficulties in interpreting cholinesterase activities determined on the whole diaphragm.

The Hydrolysis of Choline and Thiocholine Esters by Preparations of Specific and Non-specific Cholinesterase.

No quantitative comparison between the relative abilities of specific and non-specific cholinesterase to hydrolyse any particular substrate can be made until the two enzymes have been purified and their molecular weights determined. However active preparations of both enzymes hydrolyse acetylcholine and Koelle has found that acetyl thiocholine is hydrolysed rather faster than acetylcholine by the two enzymes. Koelle used a preparation of bovine erythrocytes for specific cholinesterase and purified horse serum for non-specific cholinesterase, and also made preparations from tissues of the cat. It was by no means certain that the same results would be obtained with the cholinesterases of the rat. When preparations of rat tissue were made it was found that the acetyl thiocholine was hydrolysed at a rate similar to that of acetylcholine by both specific and non-specific cholinesterase (Table VI).

TABLE VI.—*Comparison of Rates of Hydrolysis of Acetylcholine and of Acetyl Thiocholine by Preparations of Rat Specific and Non-specific Cholinesterase.*

	Enzyme preparation:			
	Non-specific cholinesterase.		Specific cholinesterase.	
Substrate.	Heart.	Harderian gland.	Brain.	Washed erythrocytes.
Acetylcholine .	57·5	86·3 .	118·8	7·71
Acetyl thiocholine	63·3	72·5 .	122·4	7·88

Acetylcholine measurements were made at pH 7·6 and a substrate concentration of 0·015M. Acetyl thiocholine measurements made at pH 6·0 and substrate concentration of 0·005 M. Results expressed as μl of CO_2 evolved/min./g. tissue or 0·5 ml. whole blood.

Return of Cholinesterase Activity In Vivo

The rate of recovery of cholinesterase activity in the rat diaphragm *in vivo* after acute poisoning with paraoxon and TEPP is rapid for the first few days and then decreases (Fig. 4). Recovery after DFP is initially slower but is more sustained. These curves show the combined effects of the recovery of specific and non-specific cholinesterase. The relative contributions to this recovery of reversal of inhibition and of resynthesis of the enzymes cannot be determined on this basis but something can be done to assess the part played by the two enzymes.

In Fig. 5 the individual recoveries of specific and of non-specific cholinesterase are expressed as percentages of the total hydrolytic effect of the two enzymes in the normal diaphragm. On this basis complete recovery of the specific enzyme is at the level of 67 per cent of total cholinesterase activity, and the non-specific enzyme at about 33 per cent (Table IV). After poisoning with paraoxon the rate of recovery of non-specific cholinesterase is relatively fast and is nearly complete in 2 days, whereas recovery after DFP is very much slower. With specific cholinesterase recovery after paraoxon is similar to recovery after DFP. Much of the difference in the recovery curves for total activity after paraoxon and

DFP shown in Fig. 4 appears to be due to the differences in recovery rates of non-specific cholinesterase after these two inhibitors.

After poisoning with paraoxon the recovery of specific cholinesterase activity over the first two days is not inferior to that of non-specific cholinesterase when the activity is measured as the ability to hydrolyse acetylcholine in strictly

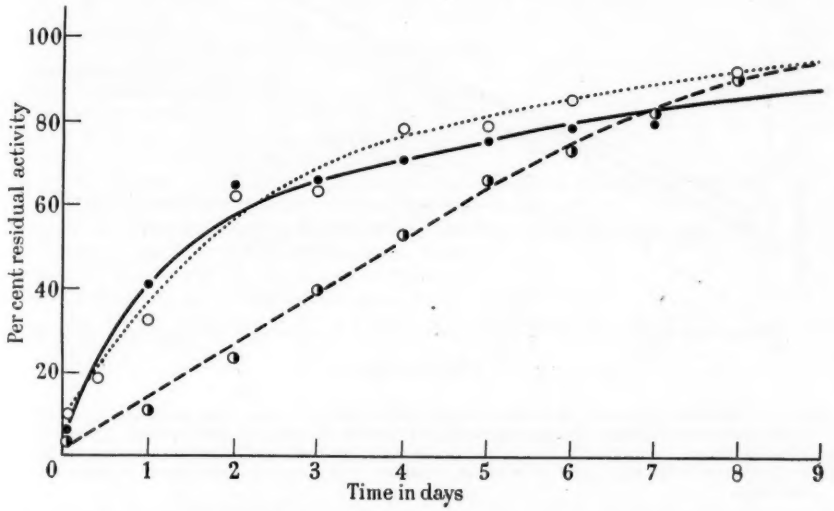

FIG. 4.—Recovery of total cholinesterase activity *in vivo* after paraoxon (●—●), TEPP (○ · · · ○) and DFP (◑ - - - ◑) (each point is the mean of 3 or 4 estimations).

comparable units (Fig. 5). This comparison has been made to examine the hypothesis that the survival of the animal is due to the rapid recovery of non-specific cholinesterase both *in vivo* and *in vitro* after paraoxon. There does not seem to be any need to invoke this hypothesis because the return of specific cholinesterase activity seems to be at least as adequate to explain recovery of the animal. Further, much of the non-specific cholinesterase in the diaphragm appears to be generally distributed throughout the muscle rather than at the myoneural junctions. Also, after DFP, provided that a lethal dose has not been given, symptomatic recovery is possible with very little return of non-specific cholinesterase activity.

DISCUSSION.

When a rat is given a lethal dose of paraoxon or TEPP the level of cholinesterase at the myoneural junctions is lowered to about 5 per cent of the normal value. If the rat is resuscitated by artificial respiration the level rises to 10 to 15 per cent, a level which is also adequate for normal function in the isolated diaphragm preparation (Barnes and Duff, 1953). With DFP, death is usually

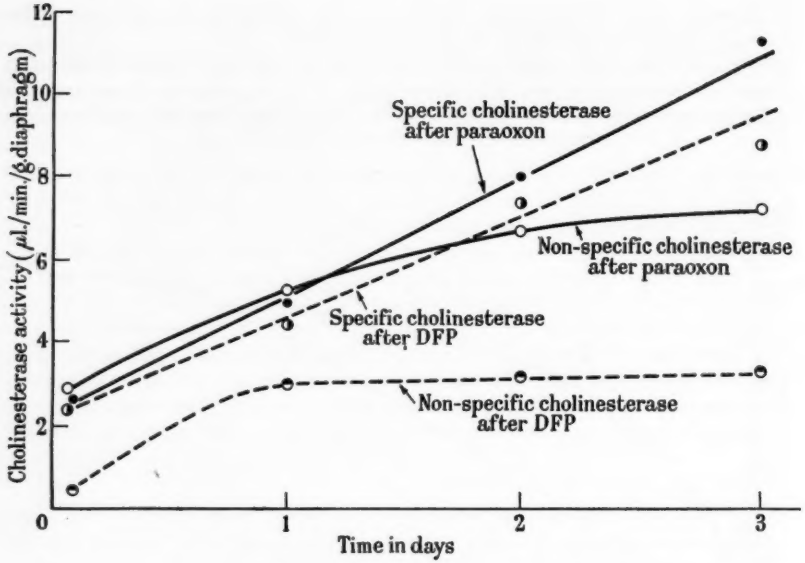

FIG. 5.—Recovery of specific and non-specific cholinesterase *in vivo* after paraoxon and DFP (substrate-acetylcholine : inhibitor-mipafox 10^{-7} M for 30 min. to give activity of specific cholinesterase. Non-specific cholinesterase activity obtained by difference between specific activity and total activity in absence of inhibitor. Each point is the mean of three estimations).

associated with levels below 5 per cent and survival with levels above 10 per cent, but this difference cannot be bridged by artificial respiration. These events can be related to the demonstration that the inhibition of specific cholinesterase in the diaphragm is partially reversible *in vitro* after paraoxon and TEPP but not after DFP. Davison (1953) has made similar observations with preparations of rat erythrocyte and rat brain cholinesterase. The reappearance of a small amount of cholinesterase activity during artificial respiration appears to be due to the dissociation of the enzyme-inhibitor complex. With DFP this dissociation does not occur to any appreciable extent and resuscitation is difficult or impossible. Other workers have attributed recovery of function after poisoning with hexaethyl tetraphosphate (Dayrit, Manry and Seevers, 1948) and TEPP (Hobbiger, 1951 ; Douglas and Matthews, 1952) to dissociation of the enzyme-inhibitor complex.

A secondary problem has been raised by the presence of non-specific cholinesterase in the diaphragm. This has led to difficulties in interpretation of results, but it appears from histochemical studies that the nonspecific cholinesterase is widely spread throughout the muscle whereas specific cholinesterase is mainly localized at the myoneural junctions. There is some non-specific cholinesterase at the myoneural junction but its activity is small in comparison with that of specific cholinesterase and there is no evidence that it plays any part in the recovery of function in the poisoned diaphragm.

SUMMARY.

In rats poisoned with TEPP and paraoxon, recovery after artificial respiration is associated with a rise in cholinesterase activity of the diaphragm from the lethal level of 5 per cent of normal to 15 per cent. This increase in activity appears to be due to reversal of inhibition. In poisoning with DFP this reversal has not been detected and artificial respiration is not effective.

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DETECTION OF COMPLETE AND INCOMPLETE ANTIBODIES TO EGG ALBUMIN BY MEANS OF A SHEEP RED CELL-EGG ALBUMIN ANTIGEN UNIT.

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A METHOD has already been described for the detection of complete and incomplete antibodies to soluble protein antigens using an ox red cell-protein antigen unit (Coombs, Howard and Mynors, 1953). The construction of such a stable red cell-antigen unit was made possible by the special nature of the ox red cells employed (Coombs, Gleeson-White and Hall, 1951). In the original work the method was used to detect antibodies to egg albumin and horse dandruff protein in human and animal sera. It has since been applied by Britton and Coombs (1954, in preparation) for the examination of the sera of hayfever patients for antibodies to the protein of Timothy grass pollen. It has also been used in an examination of sera from apparently normal persons for antibodies to egg albumin, lactalbumin and horse dandruff protein. From the experience gained in these studies it may be concluded that the method is sensitive, specific and reliable. However, the procedure is lengthy and the method, although detecting both complete and incomplete antibodies, fails to differentiate between them.

Already in the previous paper (Coombs *et al.*, 1953, Fig. 1) a further development of the method was envisaged whereby sheep cells would be used in the construction of the cell-antigen unit. This modification would result not only in a shorter test but also in one capable of differentiating a potentially precipitating antibody from an incomplete antibody. This development awaited the production of satisfactory rabbit sheep-cell non-agglutinating antibody to which the desired protein antigen could be coupled by chemical means. Tyler (1945) reported that although photo-oxidation of antisera to cellular antigens destroyed the agglutinating power of the antibodies their specific combining capacity was retained. Tyler, Fiset and Coombs (1954) showed that the Forssman antibody in the globulin fraction of a rabbit anti-fowl red cell serum after photo-oxidation had no agglutinating properties for sheep cells but possessed strong sensitizing capacity for these cells as could be shown by the anti-globulin sensitization test (Coombs, Mourant and Race, 1945). Such a photo-oxidized antibody has been coupled by chemical means to egg albumin and used for the construction of a sheep red cell-egg albumin antigen unit.

In the present paper such a cell-antigen unit has been used to examine sera for egg albumin antibodies of both the complete and incomplete types. Potentially precipitating antibodies to egg albumin agglutinate the red cell-egg albumin antigen unit while incomplete antibodies to egg albumin, although failing to agglutinate the unit directly, combine with the cell-antigen unit and may be put in evidence by the anti-globulin sensitization test.

MATERIALS AND METHODS.

Materials for the Preparation of the Sheep Red Cell-Egg Albumin Antigen Unit.

Sheep red cells.—Defibrinated blood was obtained twice weekly and stored at + 4°. Cells were washed daily for laboratory use.

Egg albumin.—This was prepared as in our previous paper (Coombs, Howard and Wild, 1952).

Rabbit anti-Forssman serum.—Advantage was taken of the fact that the red cells of the fowl contain the Forssman antigen. Four rabbits received two courses of injections with washed fowl red cells. Each injection consisted of 1 ml. of a 10 per cent suspension of washed cells in saline. Each course consisted of six injections at 2–3 day intervals. The animals were bled for serum on the 10th day after the end of the first course of injections and on the 8th day after the end of the second course. The second course sera from the four rabbits were pooled.

Preparation of the globulin fraction from the second course Forssman antiserum.—The total globulin fraction was precipitated from the unheated serum by adding to the serum an equal volume of saturated ammonium sulphate. The precipitating mixture was left overnight in the cold. The following day the material was centrifuged and the precipitate was washed once in a 50 per cent saturated ammonium sulphate solution. The precipitate was re-centrifuged and dissolved in saline buffered at pH 7·2 with phosphate buffer. The globulin solution was dialysed against buffered saline until free of NH_4 ions then stored at − 20° till required. The protein concentration was adjusted to 2·5 per cent.

Photo-oxidation of the Globulin Fraction of the Rabbit Forssman Antiserum.

The procedure adopted for photo-oxidizing the globulin solution has already been described (Tyler *et al.*, 1954). To recapitulate, the globulin solution in the presence of the photo-sensitizer, eosin Y, was exposed to a powerful source of light in one of the flasks of an enlarged modification of the constant pressure manometer devised by Dr. M. Dixon and illustrated in Fig. 2 of his book ' Manometric Methods ' (1951).

The flasks had a capacity of about 100 ml. and the side arm pipette a capacity of about 12 ml. Ground glass taps (including one between the two arms of the U-tube) were built in so that once the flasks had been mounted oxygen could be flushed through the apparatus. The apparatus was immersed in a Warburg bath at 23°. The light source consisted of a special 12 v. 239 w. bulb set in a powerful reflector. It had previously served as an aeroplane landing light. It was focussed so that its beam fell obliquely on the flask from above and maximal reflection was obtained by placing mirrors in the water bath both beneath and beside the flask. The distance between the bulb and the flask containing the globulin solution was 82 cm. and the light intensity at this distance was 300 ft. c.

The non-agglutinating Forssman antibody to which the egg albumin was subsequently coupled was prepared under the following set of conditions: 10 ml. of the 2·5 per cent globulin solution was placed in the flask and 1 ml. of a 2·0 per cent aqueous solution of eosin added. The well inside the flask was half filled with a 10 per cent solution of potassium hydroxide. The flask was fitted to the apparatus and oxygen flushed through. The light was turned on and the exposure continued until the globulin solution had absorbed 220 $mm.^3$ oxygen/ml. After this treatment the solution was stored in the dark at − 20°. It had no agglutinating capacity for sheep cells, yet up to a dilution of 1/320 it was capable of sensitizing the cells as could be shown by the anti-globulin sensitization test.

Conjugation of Egg Albumin with the Photo-oxidized Globulin.

The conjugation of the egg albumin and the photo-oxidized globulin sheep-cell antibody solutions was effected by tetrazotised benzidine (Coombs *et al.*, 1952; Coombs *et al.*, 1953). It was found to be difficult to remove the eosin from the photo-oxidized globulin solution by dialysis, but its presence did not seem to interfere with the conjugation.

Four ml. of the photo-oxidized 2·5 per cent globulin solution (containing Forssman antibody) and 1·2 ml. of 5 per cent egg albumin were mixed in the cold, and to this was added the appropriate amount of a solution of benzidine tetrazonium chloride, calculated to give approximately 8 mol. per 2 mol. protein. Following overnight dialysis against saline the

conjugated globulin antibody solution was stored at − 20° till required for constructing a sheep red cell-egg albumin antigen unit. A solution of photo-oxidized globulin sheep cell antibody azo-benzidine-azo-egg albumin stored at − 20° has been found to best able and as effective after 9 months as when first prepared.

Rabbit and guinea-pig anti-egg albumin sera.

The rabbit serum was obtained after one course of injections, the guinea-pig sera after two courses. The sera were heat-inactivated and absorbed free of antibodies to sheep cells before being used in the tests.

Human sera.

These were from three apparently normal adults. These were also heat inactivated and absorbed with sheep cells. Many human sera other than C, B and N have been tested but these three were chosen as best illustrating the potentialities of the serological method under investigation.

Rabbit anti-human globulin serum.

This particular serum was made by injecting a rabbit with human serum and was obtained after eight courses of injections. It was inactivated and absorbed free of agglutinins with well washed (7 times) sheep cells.

RESULTS.

Agglutination of the Sheep Red Cell-Egg Albumin Unit by Precipitating Antibodies to Egg Albumin in Rabbit and Guinea-pig Antisera.

The sheep red cell-egg albumin unit was prepared by mixing 1 ml. of a 2 per cent suspension of washed sheep cells and 1 ml. of a 1/5 dilution of the photo-oxidized sheep cell antibody solution conjugated with egg albumin. After 30 min. at 37° the cells were deposited by centrifugation, washed twice with saline and re-suspended in 1 ml. of saline. This suspension constituted the sheep red cell-egg albumin antigen unit.

One drop of the suspension was then added to narrow round-bottomed tubes (7 × 50 mm.) each containing one drop of varying dilutions of rabbit or guinea-pig anti-egg albumin sera or control normal sera. These sera had previously been heated at 56° for 30 min. and absorbed free of agglutinins for sheep cells. The contents of the tubes were mixed and incubated at 37° for 1 hr. The deposited cells were then examined for evidence of agglutination.

From the results shown in Table I it may be seen that the sheep red cell-egg albumin unit is stable in normal sera but is strongly agglutinated by antisera to egg-albumin. In the construction of the red cell-egg albumin unit, the conju-

TABLE I.—*Agglutination of the Sheep Red Cell-Egg Albumin Antigen Unit (2 per cent Cell Suspension) by Precipitating Antibodies to Egg Albumin in Rabbit and Guinea-pig Antisera.*

Sera.	Serum dilutions, 1 in—				
	2	8	32	128	512
Normal rabbit	—	—	—	—	—
Rabbit anti-egg albumin	++	++	++	++	++
Normal guinea-pig	—	—	—	—	—
Guinea-pig anti-egg albumin	++	++	++	++	++

++ = strong agglutination ; — = no agglutination.

gated sheep cell antibody solution could be diluted 32 times and still render the cells susceptible to agglutination by antibodies to egg albumin. No reaction occurred if an unconjugated antibody solution was used or if an antibody solution conjugated to a protein other than egg albumin was employed.

The main object of this experiment was to show that this sheep red cell-antigen unit could be agglutinated directly by potentially precipitating antibodies to the attached antigen, an occurrence which was not possible using the ox-red cell protein antigen unit (Coombs *et al.*, 1953).

Use of the Sheep Red Cell-Egg Albumin Antigen Unit to Detect Antibodies to Egg Albumin in Human Sera.

The sheep red cell-egg albumin unit was prepared as in the previous experiment except that the cells were finally re-suspended in undiluted rabbit serum (inactivated and absorbed free of sheep cell antibodies). Three ml. of the 2 per cent suspension were prepared. The red cell-antigen suspension in 0·1 ml. amounts was then added to 0·1 ml. amounts of increasing dilutions of each human serum and the contents of the tubes mixed. For measuring direct agglutination two drops of the mixture were removed from each tube and placed in a fresh tube and incubated at 37°. The red cells, after sedimentation, were examined for agglutination. The remaining serum antigen mixtures (0·2 ml. minus two drops) were also incubated for 30 min. at 37°. The cells in each tube were then deposited by centrifugation, washed twice in saline, and re-suspended in 0·1 ml. saline. One drop of each washed cell suspension was then added to one drop of a rabbit anti-human globulin serum, which had previously been absorbed with sheep cells, to test for the adsorption of human incomplete antibody to egg albumin by the red cell-egg albumin unit.

The results are shown in Table II. Serum C had no demonstrable antibodies to egg albumin. Serum N directly agglutinated the sheep red cell-egg albumin antigen unit and therefore contained agglutinating and thus potentially precipitating antibodies to egg albumin. Serum B however failed to agglutinate the red cell-antigen unit although this serum contained an antibody which adsorbed specifically on to the red cell-egg albumin antigen unit rendering the cells agglutinable by anti-human globulin serum. The behaviour of this antibody in serum B brings it within the classification of an incomplete antibody.

TABLE II.—*Use of the Sheep Red Cell-Egg Albumin Antigen Unit (2 per cent Cell Suspension) to Detect Complete and Incomplete Antibodies to Egg Albumin in Human Sera.*

Serum.	Reaction.	Serum dilutions, 1 in— 2.	8.	32	125.	256.	512.	1000	4000	16000.	Saline.
C	Direct agglutination	—	—	—	—	—	—	—	—	—	—
N		++	++	++	++	++	+	w	—	—	—
B		w	—	—	—	—	—	—	—	—	—
C	Indirect sensitization test	—	—	—	—	—	—	—	—	—	—
N		++	++	++	++	++	++	++	+	w	—
B		++	++	++	++	++	++	+	w	—	—

++ = strong agglutination; + = moderate agglutination; w = trace agglutination; — = no agglutination.

Further tests demonstrated the specificity of the antibodies in sera N and B. The three sera were also examined for antibodies to egg albumin by Boyden's (1951) method using tanned human red cells coated with egg albumin. The egg albumin-coated cells were agglutinated by serum N but not by serum C or B.

DISCUSSION.

The shortened method described in this paper for the serological detection of incomplete antibodies to soluble protein antigens is, in our view, sound in principle. It is also simple to execute and permits the differentiation between complete and incomplete antibodies.

It is hoped that later the technicalities for the preparation of the reagents necessary for the construction of the red cell-antigen unit may be simplified and improved. Photo-oxidation of the rabbit Forssman antibody affords a suitable non-agglutinating antibody to sheep cells to which the antigen can be conjugated but is satisfactory in the empirical sense only. Once the conditions which determine the production of incomplete antibodies by an animal are understood, it may be possible to make the rabbit itself manufacture *in vivo* incomplete antibodies to the Forssman antigen.

Similarly a method more satisfactory than the use of tetrazotised benzidine for the conjugation of proteins is being sought. In this connection it has been observed that photo-oxidation of a mixture of serum globulin containing the Forssman antibody and egg albumin in equimolecular amounts results in the conjugation or complexing of the two molecules. This may be shown by the fact that sheep cells exposed to this photo-oxidised mixture and subsequently washed serve admirably as a sheep cell-egg albumin antigen unit. The possibilities of this method of complexing are being further investigated.

It is uncertain whether, in looking for specific incomplete antibodies to soluble proteins by this method, a complication will be met with certain sera due to adsorptions of a non-specific agglutinating enhancing factor ; not enough sera or systems have yet been examined. From our previous experience with the ox red cell-protein antigen unit, such a factor may be a complication when the serum to be examined is used undiluted or diluted only 2 or 4 times. With the ox cell system, however, this may be completely overcome by a previous absorption of the serum under examination by a washed suspension of ox cells sensitised by rabbit antibody and also, in the test itself, by suspending the ox cell-antigen unit in undiluted rabbit serum instead of saline. Presumably similar methods applied to the sheep cell system would overcome this complication.

The human sera N and B whose reactions are recorded in Table II, are both of interest with their high content of antibodies to egg albumin. They were from apparently normal persons. Clinically the two persons were not sensitive to eggs nor did either person give a reaction on performing scratch skin test with egg albumin. Like a great many people today they had had experience of egg-made vaccines but not more so than other persons who completely lacked any demonstrable antibody. The significance of this finding is being further investigated.

We have thought it desirable to find a name to describe this type of test in which the protein (or other soluble antigen) is linked to a red cell to make a red cell-protein antigen unit. Professor J. R. Marrack has suggested "*red cell*

linked antigen test" as a general description for this type of technique. This seems to us to have much to commend it. The word "linked" is descriptive of what is involved and helps to differentiate it from the *antigen-coated red cell techniques*, such as that of Boyden (1951) using tanned red cells.

SUMMARY.

Complete antibodies (precipitating or agglutinating) and incomplete antibodies (non-precipitating or non-agglutinating) to egg albumin in human sera may be detected and differentiated the one from the other by reaction with a sheep red cell-egg albumin antigen unit.

The sheep red cell-egg albumin unit is constructed by exposing sheep red cells to a rabbit Forssman non-agglutinating antibody which has previously been coupled by chemical means with egg albumin.

One of us (R. R. A. C.) would like to thank both the Nuffield Foundation and the Agricultural Research Council for a joint personal grant in aid of this work. Likewise M. L. F. would like to thank the National Microbiological Institute of the U.S. Public Health Service for a Fellowship during the tenure of which this work was carried out.

The authors would specially like to thank Mr. H. Barber and others for generously giving their blood for these experiments.

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CHEMICAL CONSTITUTION AND ANTI-TUBERCULOUS ACTIVITY: PART I. PRIMARY AROMATIC AMINES.

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IT is becoming increasingly evident that an understanding of the relation between chemical constitution and antibacterial activity in any given series of compounds cannot be reached without an investigation of certain physico-chemical factors, notably the percentage of compound ionised at the physiological pH (7·2), the surface area of the molecule, and its distribution between lipoid and aqueous phases. Thus Albert, Rubbo, Goldacre, Davey and Stone (1945) and Albert, Rubbo and Burvill (1949) were able to demonstrate that among acridines a pKa of about 7·8 at 20° and a molecular surface area of at least 28 sq. Å were prerequisites for high bacteriostatic activity.

The aim of the present work has been to determine whether similar correlations may provide a basis for understanding the anti-tuberculous action of primary aromatic amines. The importance of primary aromatic amines in the chemotherapy of tuberculosis was early appreciated by Kuroya (1929), and a series of these bases has been investigated by Bloch, Lehr and Erlenmeyer (1945) and Bloch, Brubacher, Erlenmeyer and Suter (1947) and by Erlenmeyer, Becker, Sorkin, Bloch and Suter (1947), Erlenmeyer, Blumer, Sorkin, Bloch and Suter (1947), Erlenmeyer, Grubenmann and Bloch (1948), Erlenmeyer, Noll and Sorkin (1949) and Erlenmeyer, Sorkin and Vogtli (1949), and recently by Wagner-Jauregg, Wagner and Vonderbank (1949), Wagner and Vonderbank (1949) and Doub and Youmans (1950). Erlenmeyer and his co-workers were able to point out that *p*-substitution of lipophilic groups increased the *in vitro* activity of these compounds, a conclusion confirmed by Doub and Youmans (1950) who also noted the drastic reduction in activity caused by the addition of serum.

The authors have now investigated a series of 45 compounds possessing the primary aromatic amine structure or related thereto.

MATERIALS AND METHODS.

Organic chemistry.—A number of the amine hydrochlorides listed in Tables I, II and III were previously unknown and were synthesised for this work to provide compounds having the desired physical properties. Their preparation is described by Baxter, Cymerman-Craig and Gilbert (1953) and Cymerman-Craig, Parker and Woodhouse (1953). All test substances were of analytical purity.

Physical chemistry.—Many of the basic strengths quoted in this paper were not previously known; their determination is described by Carswell, Cymerman and Lyons (1952), Baxter *et al.* (1953), Cymerman-Craig *et al.* (1953) and Allen, Cymerman-Craig and Diamantis (1954). The percentage of these compounds ionised at pH 7·2 has been calculated from equation 1, where pKa is the negative logarithm of the acidic dissociation constant, and equals the pH value at which the base is 50 per cent ionised.

$$\text{Percentage ionised} = \frac{100}{1 + \text{antilog}\,(\text{pH} - \text{pKa})} \quad . \quad . \quad . \quad (1)$$

Since most of these values were determined by potentiometric titration in 50 per cent aqueous alcohol (the majority of the bases being insoluble in water), a correction has been added to convert these figures to the equivalent values in water. The magnitude of this correction was obtained from the average value found in cases where pKa values have been determined both in water and alcohol, and for aromatic primary amines a value of 0·4 unit has been employed. The values thus obtained are placed in parentheses in the Tables. As pointed out by Albert *et al.* (1949), these values are subject to an error of about $\pm$ 0·2 unit, which is however of no importance in the present work at pH 7·2, except for pKa values lying between 6·2 and 8·2, where a small change in pKa can cause a large change in percentage of ionisation. The partition coefficients between liquid paraffin (B.P., 1932) and water at pH 7·2 have been determined using a spectrophotometric method for a representative number of compounds in Tables I, II and III by Cymerman-Craig and Diamantis (1953) and Cymerman-Craig *et al.* (1953).

Bacteriological.—The tuberculostatic tests were performed by the serial dilution technique using *Mycobacterium tuberculosis* H37Rv. The method was standardised by acid washing all glassware and using the modified Proskauer and Beck medium (Doub and Youmans, 1950) hereinafter referred to as synthetic medium. Ten per cent sterile ox serum was added aseptically to this (= serum-synthetic medium) when required.

The water-soluble derivatives were dissolved in sterile glass distilled water in a concentration of 0·02 M (M/50) calculated as base. The less soluble compounds were dissolved in a small quantity of alcohol or 0·1 M-HCl or NaOH, and then diluted to M/50 concentration. This solution was further diluted, usually 40 times, in the synthetic and serum-synthetic media to give an initial test dilution of M/2000. After adjusting the pH to 7·0–7·2 aseptically doubling dilutions were made so that duplicate volumes of 2·5 ml. per tube of each dilution were available for inoculation.

Inoculum.—A stockpile of dried cultures of the organism, harvested from a 4-week Löwenstein culture, was prepared and stored at 4°. To initiate finely dispersed inocula without subjecting the cells to physical trauma (*cf.* Yegian and Kurung, 1947), the contents of a dried ampoule were seeded on Löwenstein medium and incubated for 3 weeks at 37°. This parent culture was stored at 4° and for use was passed through Dubos-Davis Tween-albumin medium three times, each culture being incubated for 10 days. The third Dubos culture consisting of finely dispersed cells was inoculated by dropping pipette, 0·03 ml. quantities, into media containing drug dilutions. The final cell concentration in the drug medium was 10^5–10^6/ml. After sealing with cotton wool plugs soaked in melted paraffin wax the tubes were incubated at 37° for 14 days. The tuberculostatic end-point was read as the highest dilution *completely* inhibiting visible growth.

Bacteriostatic tests with other organisms, *Staphylococcus aureus*, *Bacterium coli* and *Pseudomonas pyocyanea*, were done in beef extract peptone water (pH 7·2–7·4) as described by Rubbo, Albert and Maxwell (1942).

RESULTS.

Simple substituted anilines.

Table I gives the results of the examination of 24 derivatives of aniline in which the primary amino group is intact. They include substances containing a wide range of substituents in the *ortho*, *meta* or *para* position to the amino group. These compounds are all weak bases, ranging in basic strength from pKa 2·25 to 5·5, so that all substances in Table I exist as undissociated molecules at pH 7·2.

Anti-tuberculous and antibacterial activity is of a low order, but two compounds, *o*-aminophenol (No. 139) and *o*-aminothiophenol (No. 170) show much greater activity than any other substances in Table I, probably due to their ability to form stable metal-complexes or " chelate " compounds with trace metals. The importance of chelation in determining antibacterial activity in the oxine series has been discussed by Albert, Rubbo, Goldacre and Balfour (1947). The isomeric *p*-aminophenol (No. 138), incapable of chelation, is seen to have the normal low activity of this series. The relatively high activity of No. 139 was also pointed out by Doub and Youmans (1950) but no explanation was offered

TABLE I.—*Substituted Anilines.*

Highest dilution expressed as 1/molarity of the substance completely inhibiting growth.

Lab. ref. number.	Substance.	R_1.*	R_2.*	R_3.*	Organisms and media. H37Rv serum synthetic medium.	H37Rv synthetic medium.	*Staph. aureus* (broth).	*Bact. coli* (broth).	pKa in water at 20°.	Per cent ionised at 20° pH 7·2.	Oil/water partition coefficient pH 7·2.
128	Aniline	H	H	H	125	125	<2000	<2000	4·65	0·28	0·75
91	*o*-Toluidine	Me	H	H	<2000	<2000	,,	,,	4·46	0·18	—
92	*m*-Toluidine	H	Me	H	<2000	<2000	,,	,,	4·76	0·35	—
93	*p*-Toluidine	H	H	Me	125	250	,,	,,	5·14	0·89	2·00
94	*o*-Chloroaniline	Cl	H	H	<2000	<2000	,,	,,	2·43	0·001	—
95	*m*-Chloroaniline	H	Cl	H	<2000	<2000	,,	,,	3·47	0·02	—
65	*p*-Chloroaniline	H	H	Cl	250	250	,,	,,	4·07	0·07	3·14
163	*p*-Iodoaniline	H	H	I	1000	1000	<500	<500	—	—	—
62	*p*-Phenetidine	H	H	OEt	500	500	<2000	<2000	5·32	1·25	—
139	*o*-Aminophenol	OH	H	H	16000	32000	4000	2000	4·3	0·12	<1
138	*p*-Aminophenol	H	H	OH	1000	4000	2000	1000	5·5	1·95	<1
170	*o*-Aminothiophenol	SH	H	H	8000	128000	8000	<2000	—	—	—
71	4-Benzylaniline	H	H	CH_2Ph	<2000	<2000	<2000	,,	(4·94)	0·56	—
50	4-Diethylaminoaniline	H	H	NEt_2	4000	16000	<2000	,,	—	—	—
51	4-1′-Piperidyl-aniline	H	H	NC_5H_{10}	2000	8000	,,	,,	(6·96)	36	1·28
72	4-*Cyclo*hexylaniline	H	H	C_6H_{11}	2000	16000	,,	,,	(5·11)	0·78	—
53	*p*-Aminoazobenzene	H	H	N:NPh	4000	8000	,,	,,	3·05	0·01	—
14	Sulphanilic acid	H	H	SO_3H	<1000	<2000	<1000	<1000	—	—	—
73	4-Aminodiphenyl ether	H	H	OPh	<2000	8000	<2000	<2000	(4·84)	0·45	—
99	4-Aminodiphenyl sulphide	H	H	SPh	<8000	16000	<4000	<4000	(3·70)	0·03	—
100	4-Aminodiphenylsulphone	H	H	SO_2Ph	2000	4000	<2000	<2000	(2·87)	0·01	—
98	4-Aminodiphenylamine	H	H	NHPh	<8000	32000	<8000	<8000	(5·64)	2·75	—
119	4:4′-Diaminodiphenylsulphone	H	H	$SO_2C_6H_4.NH_2$	8000	8000	,,	,,	(2·68)	0·01	—
24	4:4′-Diaminodiphenyldisulphide	H	H	$S.SC_6H_4.NH_2$	4000	16000	2000	<2000	(3·93)	0·05	—

* See Fig. 1 (1).

by these authors. The activities of these substituted anilines against *Staph. aureus* and *Bact. coli* are uniformly low, and reached M/4000 in only two cases, Nos. 139 and 170 (*i.e.*, those capable of chelation), indicating that the formation of chelated metal complexes is one means of introducing antibacterial activity in primary amines.

The effect of increasing the molecular surface area.

Table II consists of primary aromatic amines possessing two, three or four fused ring systems. The compounds containing two rings have activity from M/1000 to M/4000; those containing three fused rings from M/4000 to M/16000 (see Table II). On the addition of a fourth ring, however, activity no longer increases (No. 160, 2-aminochrysene and No. 179, 1-aminopyrene).

Effect of substituting the primary amino group.

Table III consists of a number of N-substituted amines derived from 4-aminodiphenyl, having the structure p-C_6H_5–C_6H_4–NR′R″ in which R′ and R″ are variable (hydrophilic or lipophilic) groups which determine the oil/water partition coefficient. The molecular surface area is approximately constant; basic strengths are again such that all the substances exist completely as the un-ionised molecules at pH 7·2; while oil/water partition coefficients at the same pH value range from 0·08 to 1550, *i.e.*, exhibit a 20,000-fold variation.

In spite of this wide variation in the magnitude of the partition coefficient, the tuberculostatic activities of the compounds in this Table all lie within the narrow range from M/2000 to M/4000. These findings suggest that the lipoid/water solubility is not important in determining the tuberculostatic activity of these un-ionised amines.

DISCUSSION.

The three factors which have been investigated in this work are: (1) the effect of nuclear substitution in the simplest aromatic primary amine, aniline; (2) the effect of increasing the flat molecular surface area of primary aromatic amines; and (3) the effect of substituting the primary amino group.

The effect of nuclear substitution in the aniline molecule is shown in Table I. The activities range from M/125 to M/4000 in presence of serum, with the exception of the *ortho*-substituted anilines capable of forming chelate compounds (No. 139 and 170).

An increase in the number of atoms in the substituent will result in enhanced numbers of van der Waals' forces tending to fix the molecule more strongly to receptor sites on the organism. In agreement with this it will be seen (Table I) that, in general, activity increases with increasing molecular weight and complexity of the substituent in the benzene ring (in *absence* of serum) from M/125 to M/32000. The presence of serum provides an alternative surface for adsorption, and this results in a lowering of the effective antibacterial concentration and hence activity. The conclusion may therefore be drawn from Table I that the activity of these substances against *Myco. tuberculosis* is *primarily* due to a van der Waals' type adsorption on non-specific receptor sites. The fact that the activity is low indicates that the metabolism of the cell is affected only when these sites are heavily saturated.

TABLE II.—*Polycyclic Primary Amines.*

Highest dilution expressed as 1/molarity of the substances completely inhibiting growth.

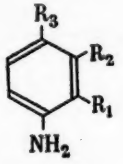
Lab. ref. number.	Substance.	Organisms and media. H37Rv serum synthetic medium.	H37Rv synthetic medium.	*Staph. aureus* (broth).	*Bact. coli* (broth)	pKa in water at 20°.	Per cent ionised at 20° pH 7·2.	Oil/water partition coefficient pH 7·2.
	(A)							
1	4-Aminodiphenyl	2000	4000	2000	<1000	4·34	0·14	55
42	3- ,,	1000	2000	2000	<1000	(4·37)	0·15	47
43	2- ,,	<2000	2000	<2000	<2000	3·84	0·04	126
44	2′-Methoxy-3-amino-diphenyl	1000	2000	1000	<1000	—	—	—
66	α-Naphthylamine	1000	2000	<2000	<2000	3·99	0·06	9·7
25	β- ,,	4000	16000	<2000	<2000	4·18	0·10	9·5
	(B)							
26	2-Aminofluorene	4000	32000	2000	<2000	(4·76)	0·35	57
57	2-Aminofluorenone	4000	8000	2000	2000	(3·07)	0·01	—
70	2-Aminodibenzofuran	4000	32000	2000	2000	(3·94)	0·05	—
122	2-Aminodibenzothiophen	8000	16000	<8000	<8000	(4·20)	0·10	—
121	9-Aminophenanthrene	16000	32000	16000	<8000	(3·97)	0·06	72
160	2-Aminochrysene	8000	16000	<8000	<8000	(3·82)	0·04	—
179	1-Aminopyrene	8000	64000	<8000	<8000	(3·64)	0·03	—

TABLE III.—*N-Substituted Amines.*

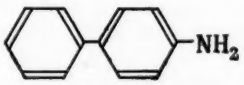
Highest dilution expressed as 1/molarity of the substance completely inhibiting growth.

Lab. ref. number.	Substance.	p-C_6H_5-C_6H_4-R; R =	Organisms and media. H37Rv serum synthetic medium.	H37Rv synthetic medium.	*Staph. aureus* (broth).	*Bact. coli* (broth).	pKa in water at 20°.	Per cent ionised at 20° pH 7·2.	Oil/water partition coefficient pH 7·2.
1	4-Aminodiphenyl	NH_2	2000	4000	2000	<1000	4·34	0·14	55
82	4-Formamidodiphenyl	NHCHO	<3000	8000	<8000	<8000	—	—	—
137	4-Acetamidodiphenyl	$NHCOCH_3$	2000	2000	<2000	<2000	—	—	—
117	N-4′-Diphenylylethylamine	NHEt	<8000	16000	<8000	<8000	(4·92)	0·50	1550
20	4-Dimethylaminodiphenyl	NMe_2	<8000	16000	<8000	<8000	(4·87)	0·47	92
174	N-4′-Diphenylylaminoethanol	$NHCH_2CH_2OH$	<8000	<8000	<8000	<8000	(4·15)	0·08	2·65
105	N:N-di-β-hydroxy-ethyl-4-amino-diphenyl	$N(CH_2CH_2OH)_2$	4000	4000	<4000	<4000	(3·60)	0·03	0·26
106	3-N-4′-Diphenylylamino-propan-1:2-diol	$NHCH_2CHOHCH_2OH$	2000	4000	<2000	<2000	(3·74)	0·03	0·085

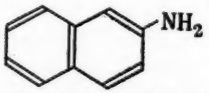
An increase in the number of fused aromatic rings will magnify the number of van der Waals' forces and increase the planar surface area of the molecules. These areas can be calculated either from Fisher-Hirschfelder atomic models or from known bond distances (Pauling, 1945). The dimensions so obtained are shown



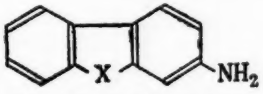
(I, Aniline)



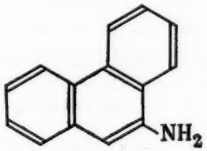
(II, 4-Aminodiphenyl)



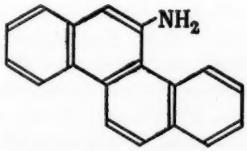
(III, β-Naphthylamine)



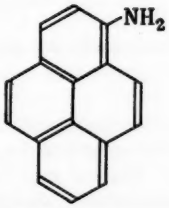
(IV ; X = CH_2, 2-Aminofluorene
X = CO, 2-Aminofluorenone
X = O, 2-Aminodibenzofuran
X = S, 2-Aminodibenzothiophen)



(V, 9-Aminophenanthrene)



(VI, 2-Aminochrysene)



(VII, 1-Aminopyrene)

FIG. 1.—Structure of compounds examined.

in Table IV, and are in close agreement with the flat areas of similarly-constituted heterocyclic aromatic bases calculated by Albert *et al.* (1949) using a slightly different method of calculation.

In Fig. 2 the tuberculostatic activities (in the presence of serum) of aniline and eleven other unsubstituted primary aromatic amines in Table II containing two, three and four rings are plotted against the flat surface area of the molecule in square Angstrom units (1Å = 10^{-8} cm.). It was decided not to include in

Fig. 2 the results for more complex substituted anilines (Table I) because of the considerable effects on van der Waals' forces of the high-molecular weight substituents in these derivatives. However, in order to obtain additional points for substances containing one ring, the three simplest *para*-substituted anilines (No. 93, 65 and 62) are included.

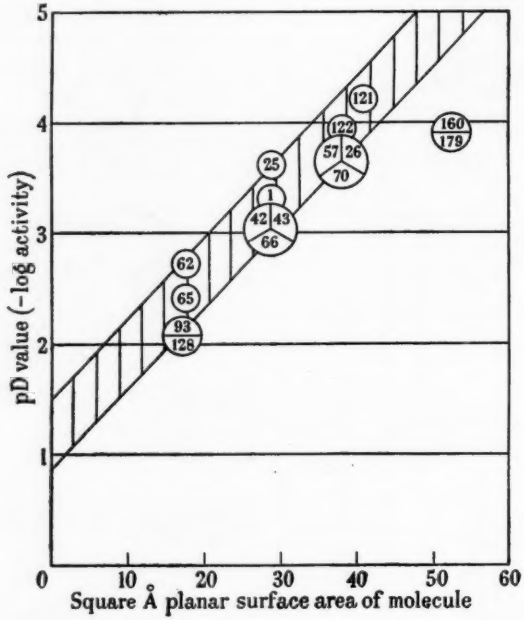


FIG. 2.—Relationship of antituberculous activity to molecular planar surface area in primary aromatic amines.

Activity is expressed in log dilutions, or pD values, where the pD value is defined as the negative logarithm of the lowest tuberculostatic molar concentration (Miller, Becker and Tainter, 1948; Feinstone, Friedman, Rothlauf, Kelly and Williams, 1947; Schild, 1947). Thus pD values of 3·00 and 4·00 mean a minimum tuberculostatic concentration of 1 : 1000 and 1 : 10000 respectively. The points represented by the individual compounds are at the centre of the circle enclosing each number.

TABLE IV.—*Planar Surface Areas of Molecules: Aromatic Ring Systems.*

Substance.	Area in sq. Å.
Benzene	17·5
Naphthalene	29·1
Fluorene, fluorenone	37·8
Anthracene, phenanthrene	40·8
Chrysene, pyrene	52·5
Benzchrysene	64·1

It is seen that the results (with the exception of No. 160 and 179) fall on or near a straight line drawn through them. This straight line has been expanded into a shaded area 0·3 unit above and below the mean values to allow for the two-fold variation in end-point (*i.e.*, ± one tube in the serial dilution method). All results (except No. 160 and 179) fall within this shaded area, and show clearly that the tuberculostatic effect of simple primary aromatic amines is directly proportional to the flat surface area of the molecule and hence to the increased number of van der Waals' forces.

In the cases of 2-aminochrysene (No. 160) and 1-aminopyrene (No. 179), activity drops to M/8000 in spite of the fact that the planar surface area of these molecules exceeds 50 sq. Å. The explanation is probably to be found in the so-called " Ferguson Effect " (Ferguson, 1939) whereby in an homologous series, a regular increase in biological activity is found until there occurs a sharp cut-off in activity when a concentration is reached when the maximum obtainable concentration in the " biophase " (*i.e.*, where biological action takes place) is less than the minimum concentration required for bacteriostasis.

It would appear, therefore, that the maximum tuberculostatic activity which can be expected in a primary aromatic amine in presence of serum will be a value of *ca.* M/16000 since any further increase in surface area apparently results in a " cut-off " in activity due to a " Ferguson Effect." In view of this comparatively low *in vitro* activity, it is unlikely that primary aromatic amines will be found effective in the chemotherapy of tuberculosis. Doub and Youmans (1950) have found nine primary aromatic amines (including No. 1, 70, 122, 139 and 163) to be ineffective *in vivo* in experimental tuberculosis in mice. Okamoto (1948) claimed high *in vivo* activity for *o*-aminophenol (No. 139) but Croshaw (1952) reported complete absence of activity in guinea-pigs.

SUMMARY.

Forty-five compounds possessing the primary aromatic amine structure or related thereto have been examined for their antituberculous activity. They have been shown to exhibit antibacterial activity of a low order.

In spite of this low level the antituberculous activity was found to be proportional to the magnitude of the total planar surface area and hence to the total van der Waals' (or surface) forces.

Variation in oil/water partition coefficient was without appreciable effect on the activity of these substances, almost completely un-ionised at pH 7·2.

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STUDIES ON THE MECHANISM OF SHOCK: THE EFFECT OF LIMB ISCHAEMIA ON TISSUE TEMPERATURE AND BLOOD FLOW.

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THE temperature fall after injury has been known for a long time (see Wiggers, 1950). Since it occurs both on the surface and in the deeper tissues of the body and is accompanied by a fall in O_2 consumption, it has been attributed to a decrease in heat production (Tabor and Rosenthal, 1947 ; Green and Stoner, 1954). It has been suggested that a fall in energy production is the prime metabolic effect of injury (Stoner and Threlfall, 1954). However, Post, Visscher and Wiggers (1948) have pointed out that the reduced O_2 consumption need not be " due to intrinsic disturbances of cellular metabolism," but could be " secondary to concomitant changes in the circulation and respiration." They tried to decide this question for haemorrhagic shock but could not reach a definite conclusion. This problem was also examined by Tabor and Rosenthal (1947), who considered that while the changes in *haemorrhagic* shock probably resulted from failure of O_2 transport this was not a major factor in *ischaemic* shock. A recent demonstration (Whiteley, Stoner and Threlfall, 1953) that the inhibition of mitosis and ^{32}P uptake in the hair follicles of the skin by limb ischaemia is due to the attendant cardiovascular disturbance led to further study of the problem.

The fall in temperature and O_2 consumption can only be attributed to a primary metabolic disturbance if adequate amounts of substrate and O_2 can be shown to be available to the tissues. The method of internal calorimetry elaborated by Grayson (1952) whereby the temperature and blood flow in certain organs can be measured in the conscious animal, provides an experimental approach to this problem. By comparison with the effects of haemorrhage little is known of the influence of limb ischaemia on cerebral and hepatic blood flow. This method also gives useful information concerning the energy metabolism of the organs in shock for, although the total O_2 consumption of the body is a function of the muscle mass (Krebs, 1950), the main centres of heat production are the brain and liver (Feitelberg and Lampl, 1935 ; Federov and Shur, 1942 ; Birnie and Grayson, 1952). Skin and rectal temperatures have been more commonly studied after injury and with the growing interest in the metabolic effects of injury it is important to relate these changes to those in the deeper organs. Rectal temperature is often a poor indicator of general body temperature (Grayson, 1951) so that its linking with O_2 consumption as a measure of general metabolic rate may well be unjustified.

METHODS.

Cross-bred male rabbits (1·5–3·5 kg. body wt.) from one source and free from coccidiosis were used. They were fed on M.R.C. diet 18 (Bruce, 1947).

The temperatures of the skin and rectum were measured with copper-constantan thermocouples attached to a Cambridge skin temperature meter. A non-growing area of skin (Whiteley and Ghadially, 1951) was chosen about the middle of the trunk just caudal to the costal margin. The thermocouple was inserted through the anus for a distance of 4–5 cm.

The temperatures of the liver and brain were determined and their blood flow assessed by the method of internal calorimetry using heated thermocouple recorders. The construction of these, their standardization, method of implantation and recording have been described in a series of papers by Grayson and his colleagues (Birnie and Grayson, 1952; Grayson, 1952; Grayson and Haigh, 1953; Grayson and Johnson, 1953; Ginsburg and Grayson, 1954) and their methods were used here with minor modifications. The recorders were varnished with Formite Damarda lacquer (Bakelite, Ltd.) as advised by Linzell (1953). A recorder was only used once and the instrument factor (F) determined in each case for a temperature increment of 1°, using a 5 per cent w/v solution of gelatin (Haigh, 1954). The mean value of F in a series of 65 recorders was 111 $\pm$ 13 (standard deviation—S.D.). In accordance with the views of Ginsburg and Grayson (1954) results are expressed in terms of the conductivity increment (δk) due to the flow of blood in the tissue around the recorder. This figure is the difference between the thermal conductivity of the living and dead tissue, measured as the square of the current required to raise the temperature of the heater filament by 1°, divided by F for the recorder. Changes in δk are considered to be directly proportional to changes in blood flow. This has been questioned by Linzell (1953) but an approximately linear relation between δk and blood flow over the physiological range has been demonstrated for the liver by Grayson (1952) and by Grayson and Haigh (1954) and for the brain by Carlyle, Field, Grayson and Rogers (1954). The mean value for the thermal conductivity of dead liver was found to be $11{\cdot}4 \times 10^{-4}$ (S.D. $1{\cdot}3 \times 10^{-4}$) in good agreement with the value given by Grayson (1952). The same value for dead brain was $10{\cdot}5 \times 10^{-4}$ (S.D. $1{\cdot}9 \times 10^{-4}$). The values of δk for normal rabbit liver and brain were $12{\cdot}8 \times 10^{-4}$ (S.D. $3{\cdot}2 \times 10^{-4}$) and $8{\cdot}7 \times 10^{-4}$ (S.D. $2{\cdot}5 \times 10^{-4}$) respectively. These values are higher than those reported by Carlyle *et al.*, (1954) the difference probably being accounted for by the use of anaesthesia in their experiments.

The temperature of the dorsal muscle and of the rectus femoris was determined with a copper-constantan thermocouple (36 S.W.G.) inserted through a wide bore needle. The temperature of the mixed venous blood was obtained with a similar thermocouple, ensheathed in polythene tubing except for the junction, which was inserted into the right atrium *via* the right external jugular vein, the wires being brought out behind the ear through a puncture in the skin. Similar thermocouples were used in acute experiments to obtain the temperature of the blood in the left ventricle by passing them down the right common carotid artery and of that in the caudal ends of the abdominal aorta and inferior vena cava which were approached through the corresponding femoral vessels. These thermocouples were connected to the recording instrument used for the liver and brain.

The various thermocouples and recorders were inserted under ether anaesthesia on the day before the experiment except for those in muscle. These latter were either inserted just before the experiment or during the ether anaesthesia for the application of the tourniquets. The cerebral recorders were inserted into the left parietal lobe through a drill hole in the skull. The liver recorders were usually placed in the middle lobe. The rabbits were allowed to recover from the anaesthetic and given access to food and water overnight. Bilateral hind limb ischaemia was produced by the method of Bywaters and Popják (1942) at about 10·00 a.m. on the morning of the experiment after a period of control observations. The tourniquets were applied during a short period ($<$ 5 min.) of ether anaesthesia and so that the first turn of the tubing formed a figure of eight about the knee joint. A 4-hr. period of ischaemia was used which, at an environmental temperature of 18–22°, is known, from these and other experiments, to give a 93 per cent mortality rate. The controls were given the same period of ether anaesthesia. Observations on temperature and blood flow were usually recommenced an hour later and continued until the rabbit died or, in the case of the controls, was killed. They were then left until the next day when the thermal conductivity of the dead tissue was determined and the position of the recorders and thermocouples verified.

The area of ischaemia was determined by injecting 2·0–3·0 ml. 20 per cent Na fluoresceinate intravenously during the first hour of the ischaemic period and examining the skinned legs of rabbits killed with the tourniquets in place in the ultra-violet light from a Hanovia "Cromatolite." This method was also used to study the return of the blood supply to the tissues after ischaemia.

The acute experiments referred to in the text were done under urethane anaesthesia, 4–5 ml./kg. body wt. of a 25 per cent (w/v) solution except after limb ischaemia when half this dose was used. The effects of haemorrhage were studied in heparinised rabbits anaesthetised in this way. They were bled from the carotid or femoral arteries into a conical flask the pressure in which could be varied by a sphygmomanometer bulb. The blood pressure in these experiments was recorded from the carotid artery with a mercury manometer.

Hexamethonium iodide (Paton and Zaimis, 1951) was given intravenously dissolved in 0·9 per cent NaCl. Dibenamine hydrochloride was prepared for intravenous injection as described by Nickerson and Goodman (1947).

When possible the results are expressed as the mean ± S.D. and a statistical comparison of the means made by Student's "*t*" test as modified by Fisher (1934) for small samples.

RESULTS.

Effect of Anaesthesia on Temperature and Blood Flow.

The normal resting temperatures of the various tissues are shown in the Table. The values for the brain and liver are higher than those recorded by Birnie and Grayson (1952) in the unanaesthetised rat. This may be a species difference as similar values to theirs were found in the rat with the apparatus used in these experiments. They also found the temperature higher in the liver than the brain, whereas in the rabbit it was about the same. The temperature of these organs was constantly higher than that of the mixed venous blood. Acute experiments with thermocouples in the right atrium and left ventricle showed that the temperature of the blood on the two sides of the heart was about the same. Any heat lost during passage through the rabbit's lung may be compensated for by heat gained from the cardiac muscle the temperature of which was found to be above that of the blood. The temperature of the resting dorsal muscles was below that of the mixed venous blood, whereas the deep muscle temperature of the rectus femoris (40·3° ± 0·1) was about the same as that of the blood, but under these conditions it was not in a completely resting state.

The temperature of all the tissues studied is lowered by ether anaesthesia. This was quite evident (Fig. 1 and 2) although the duration of anaesthesia was much shorter than in the experiments of Birnie and Grayson (1952) and although the recordings were not recommenced until 1 hr. after withdrawal of the ether. It was especially so in the brain, liver and muscle where the temperature was still somewhat below the resting level even after 7–8 hr. Recovery was taking place throughout this period, but it was very slow and was not complete till the following morning. The fluctuations in the blood temperature followed those in the liver and brain (Fig. 1). The skin and rectal temperatures were more variable and although they were usually depressed during the period studied, the falls were not so great, occasional rises were seen and recovery occurred sooner (Fig. 2). The maximum changes in temperature during the 2nd to the 4th hr. after short ether anaesthesia are shown in the Table. Increasing the period of anaesthesia to 9 min. caused a greater initial drop in temperature but it had still largely recovered after 4 hr. (Fig. 3).

Blood flow through the liver is depressed by ether, but the effect is short-lived (Birnie and Grayson, 1952). In these experiments the flow in the brain and liver

had usually returned to the pre-anaesthetic level when observations were recommenced. The fluctuations during the period of observation are shown in Fig. 1 and 3, the average variations from the mean pre-anaesthetic level being given in the Table.

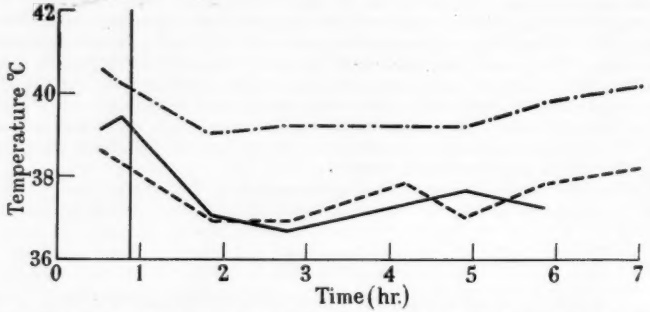

FIG. 1.—The effect of a 4–5 min. period of ether anaesthesia on the temperature of the brain (·—·—·), rectum (———) and skin(- - - -) of a rabbit. Period of anaesthesia indicated by vertical line. Environmental temperature 19°.

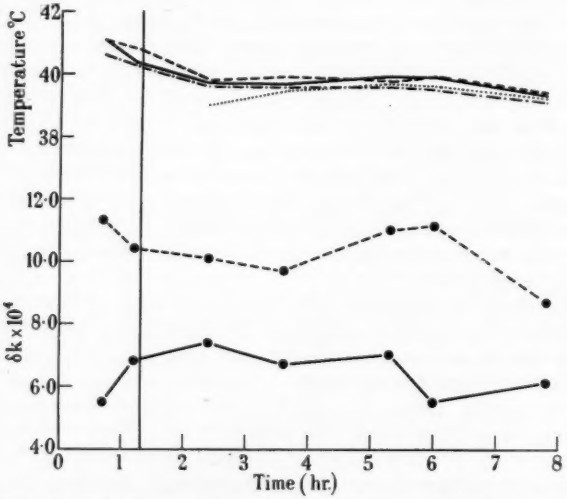

FIG. 2.—The effect of a 4–5 min. period of ether anaesthesia on the temperature of the brain (———), liver (- - - -), dorsal muscle (. . . . .) and mixed venous blood (·—·—·) of a rabbit. The blood flow (δk) in the brain (·———·) and liver (· - - - - ·) is shown in the lower part of the figure. Period of anaesthesia indicated by vertical line. Environmental temperature 22°.

Temperature Changes after Death.

To have a standard against which to compare the temperature changes in shock, normal rabbits were killed by cervical dislocation and the rate of heat loss studied. In the absence of oxidative heat production the temperature of the

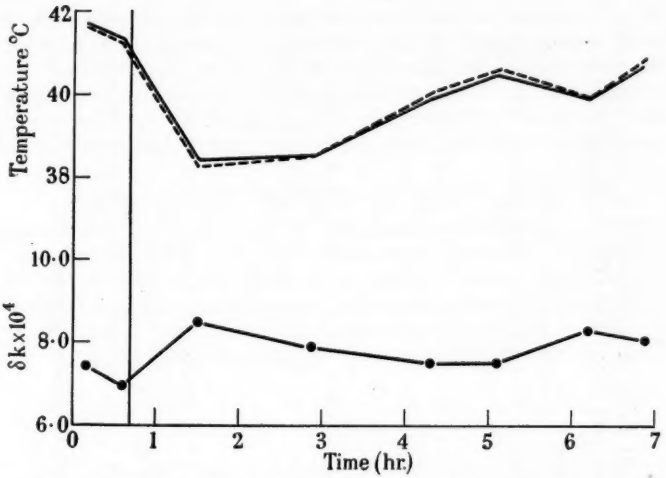

FIG. 3.—The effect of a 7·5 min. period of ether anaesthesia on the temperature of the brain (——) and liver (- - - -) of a rabbit. The blood flow (δk) in the brain is shown by the bottom curve (·——·). Period of anaesthesia indicated by vertical line. Environmental temperature 20°.

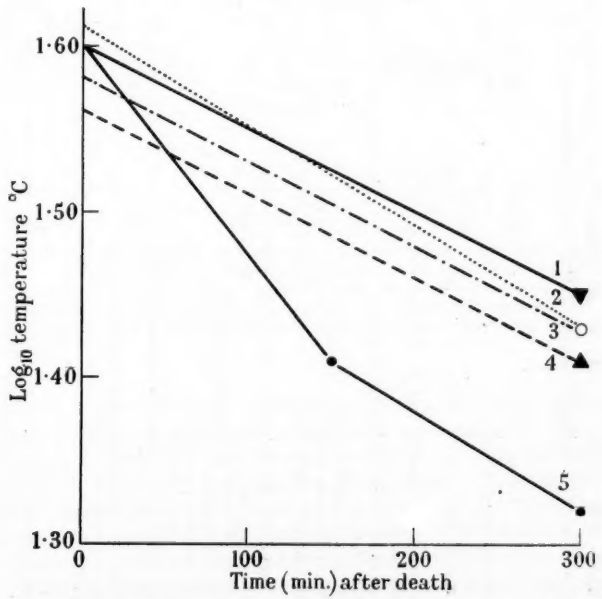

FIG. 4.—The post-mortem temperature changes in rabbit tissues; 1 = muscle, 2 = liver, 3 = rectum, 4 = skin and 5 = brain. Each curve represents the mean of 3–6 experiments. Environmental temperature 19°.

TABLE—*The Effect of (A) Ether Anaesthesia (4–5 min.) alone and (B) Ether Anaesthesia (4–5 min.) followed by a 4 hr. Period of Bilateral Hind Limb Ischaemia on the Temperature and Blood Flow (δk) in Rabbit tissues.*

Tissue.	Brain.	Liver.	Mixed venous blood.	Dorsal muscle.	Rectum.	Skin.
Normal resting temperature	40·4 ± 0·6 (48)	40·5 ± 0·6 (50)	40·0 ± 0·5 (7)	39·6 ± 0·4 (5)	38·9 ± 1·0 (18)	37·2 ± 1·1 (19)
Normal resting $\delta k \times 10^4$	8·7 ± 2·5 (21)	12·8 ± 3·2 (30)	—	—	—	—
(A) Max. temp. fall during 2nd to 4th hr. after ether	0·8 ± 0·5 (11)	1·2 ± 0·7 (12)	1·0 (2)	2·6 (2)	0·9 ± 0·4 (6)	1·6 ± 1·0 (6)
Change in $\delta k \times 10^4$ during 2nd to 4th hr. after ether :						
Max. increase (aver.)	1·3	2·5	—	—	—	—
Max. decrease (aver.)	3·4	2·8	—	—	—	—
Temp. 4 hr. after ether	39·8 ± 1·0 (11)	39·8 ± 0·6 (12)	39·4 (2)	38·7 ± 0·9 (5)	39·6 ± 0·6 (6)	37·1 ± 0·6 (6)
(B) Max. temp. fall during 2nd to 4th hr. of limb ischaemia	3·1 ± 0·8* (26)	3·1 ± 0·6* (23)	3·1 ± 0·4 (4)	2·1 (3)	2·8 ± 1·1* (10)	2·4 ± 1·3 (12)
Change in $\delta k \times 10^4$ during 2nd to 4th hr. of limb ischaemia :						
Max. increase (aver.)	1·3	1·6	—	—	—	—
Max. decrease (aver.)	2·3	2·5	—	—	—	—
Temp. at end of 4 hr. limb ischaemia	38·1 ± 0·8* (23)	37·8 ± 0·9* (19)	37·5 ± 0·8 (5)	37·9 ± 0·7 (3)	35·7 ± 1·2* (9)	35·1 ± 0·9* (11)
Per cent of rabbits showing temp. recovery during ischaemia	92	73	100	100	20	70
Temp. at death	32·5 ± 2·0 (18)	33·5 ± 1·0 (14)	34·0 ± 1·9 (4)	34·8 ± 1·9 (3)	30·9 ± 2·9 (7)	31·8 ± 2·9 (7)

Where possible these figures are the means ± standard deviation, the number of animals being shown in parentheses.
* = Significantly different from corresponding control mean at $P<0·01$.

tissues fell exponentially and the log. temperature-time curves (Fig. 4) obtained must represent the maximum possible rate of fall under present environmental conditions. Temperature in the liver and brain fell significantly faster ($P < 0{\cdot}05$) than in the dorsal muscle, skin and rectum which all lost heat at the same rate. The temperature of the brain fell very rapidly during the first 150 min. after death and then rather more slowly, at about the rate for the liver.

Temperature and Blood Flow during Limb Ischaemia.

In confirmation of the findings of Macfarlane and Spooner (1946) the use of Na fluoresceinate showed that the tourniquets produced complete ischaemia from about the middle of the thigh downwards. Above that point the muscle beneath the tourniquets usually stained although sometimes patchily. The temperature in the rectus femoris reflected this and fell rapidly in both parts of the muscle (Fig. 5). This lasted for about 2 hr. after which the temperature remained

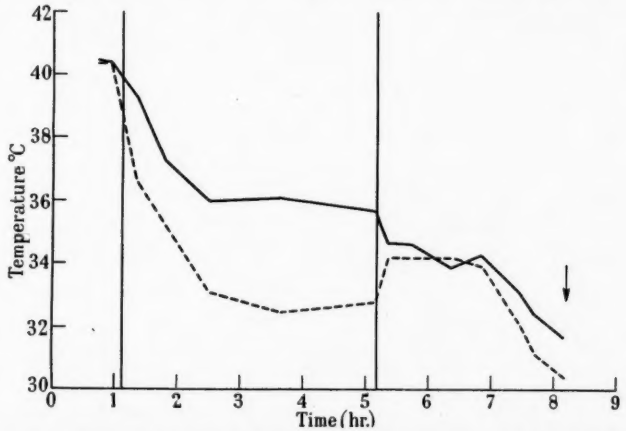

FIG. 5.—The effect of bilateral hind limb ischaemia on the temperature in the middle (———) and lower (- - - -) third of the rectus femoris of one side. Beginning and end of the ischaemic period indicated by the vertical lines. Time of death shown by the arrow. The rabbit was given 1·0 ml. 20 per cent Na fluoresceinate/kg. body wt. just after removal of the tourniquets, and at autopsy the muscle around the thermocouples fluoresced as did the rest of the muscles on both sides.

steady with none of the tendency to recovery during the latter part of the ischaemic period seen in the rat when the circulation was occluded with metal clamps (Stoner and Green, 1948). There was a good deal of variation in the final temperatures and those in Fig. 5 were higher than usual. Commoner temperatures were 33° for the upper part of the muscle and about 27° for the lower part. Part of this variation was due to the different thickness of the muscle at the two sites and to differences in the depth of the thermocouples. There is a steep temperature gradient between the deep part of the muscle and the subcutaneous tissue. The temperature of the latter fell to very low levels during ischaemia, but the same difference between the upper and lower parts of the thigh was observed; *e.g.*, in one experiment, the temperature of the subcutaneous tissue at the junction of

the upper and middle thirds was 24·3° after 4 hr. ischaemia, while that of the lower third was 18·4°.

The pattern of the temperature and blood flow changes in the other tissues during limb ischaemia is shown in Fig. 6–8. In most tissues the temperature fall was significantly greater than in rabbits given ether alone and this was reflected in the temperature of the mixed venous blood (Table). Although the mean temperatures at the end of the ischaemic period were significantly lower

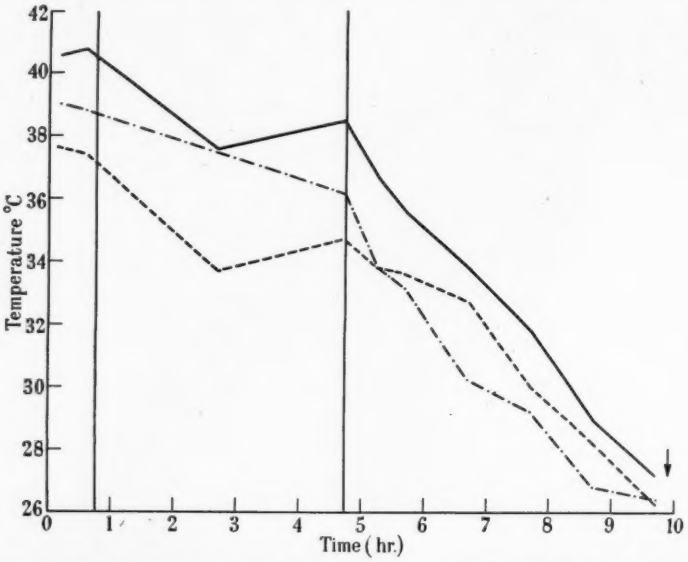

FIG. 6.—The effect of bilateral hind limb ischaemia on the temperature of the brain (———), rectum (·—·—·) and skin (- - - -) of a rabbit. Beginning and end of the ischaemic period indicated by the vertical lines. Time of death shown by the arrow. Length of ether anaesthesia for application of tourniquets 5 min. Environmental temperature 18–19°.

than in the corresponding controls, some recovery had occurred (Table). The greater temperature changes could not be correlated with alterations in blood flow (Fig. 7, 8) for the changes were the same as in the controls (Table). The tendency for the tissue temperatures to recover towards the end of the ischaemic period could be prevented by increasing the duration of the initial exposure to ether to 8–9 min.

Temperature and Blood Flow after Limb Ischaemia.

The rabbits survived for 2 hr. 34 min. ± 79 min. after removal of the tourniquets.

The temperature of the hind limb might be expected to rise rapidly when the circulation was restored as after non-fatal unilateral hind limb ischaemia in the rat (Stoner and Green, 1948). However, a marked rise in muscle temperature was not seen after these lethal periods of bilateral hind limb ischaemia (Fig. 5). The muscle temperature remained constant for some time, about a degree above the

lowest temperature reached during the ischaemia and then, quite suddenly, fell progressively until the rabbit died. This lack of recovery did not appear to be due to continuation of the ischaemia through, for example, intravascular thrombosis. Some blood at least was passing through the damaged tissue. The arteriovenous temperature difference between the blood in the abdominal aorta at its bifurcation and in the inferior vena cava at the junction of the two iliac veins was increased. When Na fluoresceinate was given intravenously after removal of the

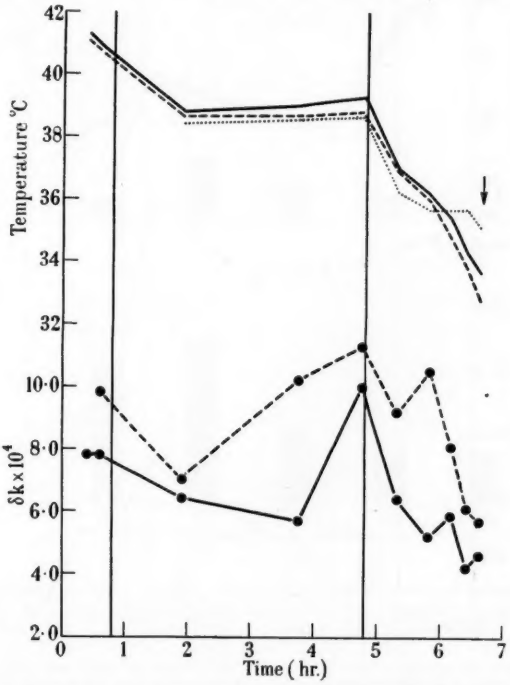

FIG. 7.—The effect of bilateral hind limb ischaemia on the temperature of the brain (——— liver (- - - -) and dorsal muscle (. . . . .) and on the blood flow (δk) through the brain (·——· and liver (·- - - - ·) of a rabbit. Beginning and end of ischaemic period indicated by the vertical lines. Time of death shown by the arrow. Length of ether anaesthesia for application of tourniquets 4 min. Environmental temperature 21°.

tourniquets, except for occasional small patches, all the hind limb muscles fluoresced well at autopsy. If the rabbit was killed at the end of the ischaemic period and the isolated hind quarters perfused through the abdominal aorta with Krebs and Henseleit's (1932) Ringer's solution, oxygenated but without glucose, at a temperature of 38–39° and a pressure of 100 mm. Hg, the muscle temperature rose rapidly. In one case the increase was from 32·7° to 38° in 40 min.

The temperature of the other tissues fell immediately the tourniquets were removed (Fig. 6–8). The temperature usually fell most rapidly in the brain, but there was little difference between it and the liver, rectum and dorsal muscle,

the mean temperature falls during the first hour after removal of the tourniquets being :—brain 2·4° ± 0·9 ; liver 2·1° ± 0·8 ; rectum 2·1° ± 1·2 ; dorsal muscle 2·3° ± 1·5. This fall continued in the brain, liver and rectum until death. In the dorsal muscle, after the initial fall, the temperature remained fairly constant until shortly before death (Fig. 7). The mixed venous blood temperature also fell, but no longer followed the changes in the brain and liver. The brain tem-

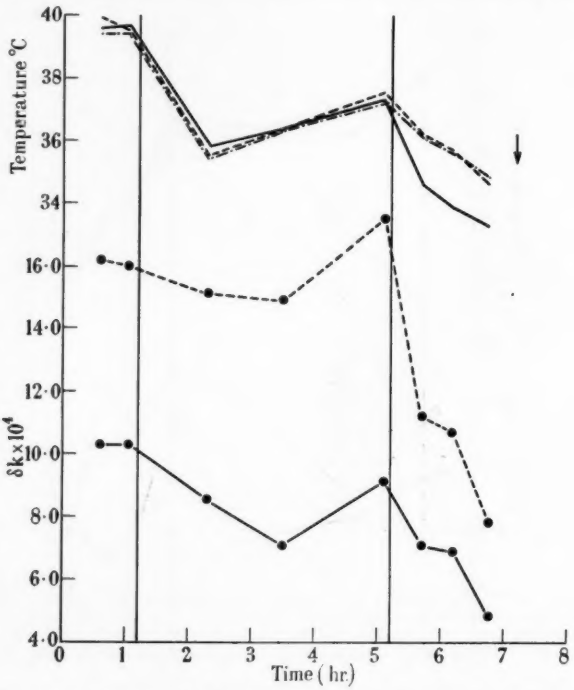

FIG. 8.—The effect of bilateral hind limb ischaemia on the temperature of the brain (———), liver (- - - -) and mixed venous blood (·—·—·) and on the blood flow (δk) through the brain ·——·) and liver (·- - - -·) of a rabbit. Beginning and end of the ischaemic period indicated by the vertical lines. Time of death shown by the arrow. Length of ether anaesthesia for application of tourniquets 3·5 min. Environmental temperature 20°.

perature soon fell below that of the blood, and at death the latter temperature was above that of both brain and liver (Fig. 8, Table). This dissociation usually occurred when the organ temperatures fell below 36°. As previously reported (Whiteley *et al.*, 1953), the temperature changes in the skin of the trunk were least striking and most variable. The average fall during the first hour was 1·1° ± 0·7 after which the temperature remained stationary for a time. This plateau was usually more evident than in Fig. 6, and it was not usually until within the last hour of life that the temperature fell steeply.

The pattern of these temperature changes in the dorsal muscle and skin is obviously different from that found after death from cervical dislocation. Simi-

larly, although the initial temperature fall was greatest in the brain, it was little more than half that which occurred in the first hour post mortem and was not strikingly different from the fall shown by other organs. The initial temperature changes in the liver, rectum and dorsal muscle were much nearer to those seen in the first hour after death. However, heat loss did not proceed exponentially and the temperature-time curves for these organs after release of the tourniquets

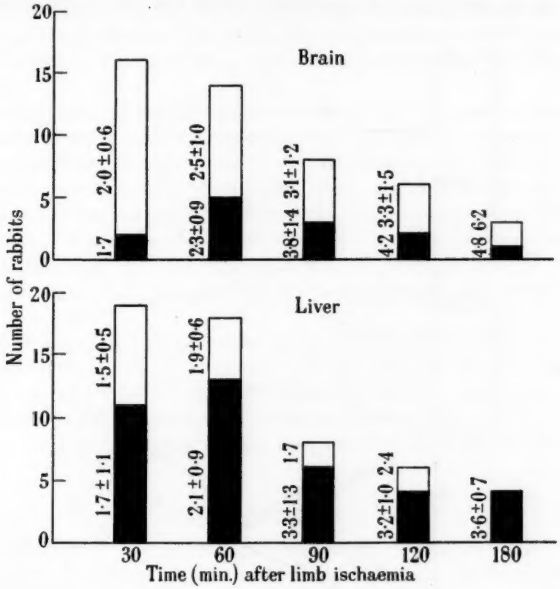

FIG. 9.—The histogram shows the number of rabbits in which the blood flow in the brain and liver was reduced below an arbitrary point (see text) when examined at intervals after removal of the tourniquets. The height of the columns shows the number of animals studied and the shaded portions show the number in which δk for the brain was reduced by more than $3{\cdot}4 \times 10^{-4}$ and the liver by more than $2{\cdot}8 \times 10^{-4}$ below the mean pre-ischaemic level. The figures at the side of the columns give the temperature fall (°C. $\pm$ S.D.) from the level immediately before release of the tourniquets. Statistical comparison of the means ("t" test) at 60 and 90 min. after release for the brain and at 30 and 60 min. for the liver showed that at these times there was no significant difference between the temperature fall in those animals with a reduced blood flow and those where the flow was within normal limits.

approximated more closely to straight lines. Energy production is, therefore, continuing although at a reduced rate.

At death the temperatures were invariably low (Table), but they varied considerably and there did not appear to be any critical temperature associated with death. Rabbits can withstand much lower body temperatures than these (Cohnheim, 1890; Ariel, Bishop and Warren, 1943). In most cases the longer the survival time the lower the final temperature, *e.g.*, final brain temperature in Fig. 6 and 7.

The blood flow through the brain and liver also fell when the tourniquets were removed (Fig. 7, 8). In both cases the fall was irregular and quite large fluctuations were seen. The hepatic blood flow was more affected and the mean per cent

reduction in δk just before death was 35 per cent for the brain and 57 per cent for the liver. A histogram was constructed (Fig. 9) to show the proportion of animals, examined at intervals after removal of the tourniquets, in which δk had fallen below the pre-ischaemic level by more than $3{\cdot}4 \times 10^{-4}$ in the brain and by more than $2{\cdot}8 \times 10^{-4}$ in the liver. These criteria were adopted because they represent the maximum reductions seen in the control animals (Table). The histogram also gives the mean temperature fall in the brain and liver at the differ-

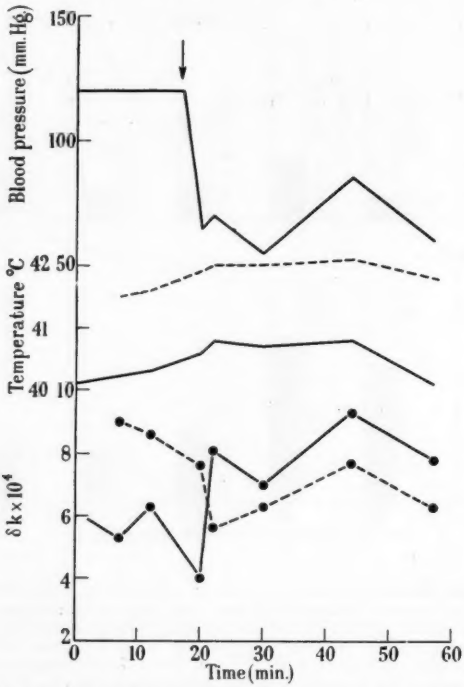

FIG. 10.—The effect of a sudden haemorrhage from the femoral artery (↓) on the blood pressure, liver (- - - -) and brain (———) temperature and liver (· · · · · ·) and brain (·———·) blood flow (δk) in a rabbit under urethane anaesthesia.

ent times after release of the tourniquets in those rabbits in which the blood flow was reduced and in those in which it remained within normal limits. It is clear that the blood flow through the liver suffers an earlier and more frequent reduction than that through the brain. Eventually the blood flow through both organs is affected and only 2 rabbits died without showing some, usually considerable, reduction in cerebral and hepatic blood flow. It is also clear that for the first 90 min. after release of the tourniquets the same fall occurred in the brain temperature whether the blood flow was reduced or not. The same was true in the liver for the first 60 min. after the ischaemic period. The differences between the mean shown in Fig. 9 during these periods are not statistically significant. After 60

min. the fall in hepatic blood flow seems to have some influence on the fall in liver temperature, but the numbers were insufficient for a statistical comparison.

The mechanism of these changes in blood flow has not been elucidated, but a few additional experiments were performed. To investigate the possibility that the opening of the vascular bed in the hind limbs when the tourniquets were removed might act like a sudden haemorrhage, the effect of the latter was observed in the anaesthetised rabbit (Fig. 10). The main difference from the changes after limb ischaemia is the transient rise in organ temperature which accompanies the fall in blood pressure. This confirms a previous observation by Grayson (personal communication).

In an attempt to cause further dissociation of the changes in temperature and blood flow efforts were made to improve the circulation through the liver and brain. A few experiments were done in which dibenamine HCl (15 mg./kg. body wt.) or hexamethonium iodide (5 mg./kg. body wt.) were given intravenously either shortly before the tourniquets were removed or later when changes in blood flow had occurred. Apart from a short-lived increase in cerebral blood flow after hexamethonium these drugs had little effect in these experiments, and it was not possible to accentuate the dissociation of temperature and blood flow by this means.

In view of the possible rôle of bacterial factors in shock (Fine, Frank, Schweinburg, Jacob and Gordon, 1952) a rabbit (1·5 kg.) was given two oral doses of 300 mg. terramycin on the day of the implantation of the recorders and a further 300 mg. just before the tourniquets were applied. The survival time in this rabbit was 1 hr. and the pattern of the temperature and blood flow changes was unaltered.

DISCUSSION.

The main purpose of this investigation was to determine whether the fall in tissue temperature in shock was due to an intrinsic metabolic derangement independent of blood flow change or was secondary to lowered availability of substrate and O_2 following a reduction in blood supply. By the method of internal calorimetry it has been possible to determine the temperature and thermal conductivity of the liver and brain of unanaesthetised rabbits in shock after a period of what proved to be incomplete hind limb ischaemia. In this method the changes (δk) in thermal conductivity are equated with changes in blood flow. Its limitations are adequately considered in the writings of Grayson and his colleagues, but it should be emphasized that δk is not a measure of the total blood flow through the organ, but of the flow through a collar of tissue around the recorder of less than 5 mm. radius. However, as this is also the tissue used for temperature measurement it is not altogether a disadvantage for the present purpose, although it makes direct comparison with the results of other blood flow methods difficult.

The changes in liver and brain temperature during the production of the injury are unaccompanied by significant changes in blood flow. The metabolic depression produced by the anaesthetic seems to be increased and prolonged. In view of the greater sensitivity to anaesthesia after severe injury the elucidation of this phenomenon might prove of clinical importance.

The changes after injury are more complex with a reduction in both temperature and blood flow in the brain and liver. Similar alterations in hepatic blood flow

were described in haemorrhagic shock by Johnson (1953), Heinemann, Smythe and Marks (1953) and Friedman, Milrod, Frank and Fine (1953) and in traumatic shock by Seneviratne (1949) using direct microscopy of the liver edge. Less attention has been paid to the cerebral blood flow but changes of the type described here are implied in the results of Gibson, Seligman, Peacock, Fine, Aub and Evans (1947). It is obviously important that the cerebral blood flow is better sustained than that to the liver. Of the more commonly recorded skin and rectal temperatures only the latter gave any indication of internal temperature. With the severe restriction of the circulation in the skin of the trunk in this form of shock (Whiteley *et al.*, 1953) a greater fall in its temperature might have been expected, but the changes appear to be dominated by those in the underlying tissues. Similar experiments on rats (unpublished) have shown that there is no real correspondence between the changes in the rectal temperature and those in the brain and liver; in rats the fall in rectal temperature considerably exceeds that in the liver and brain.

The behaviour of the uninjured muscle temperature is also interesting. Delorme (1953) has described the gradual accumulation of erythrocytes in muscle in haemorrhagic hypotension although his results seem at variance with the earlier dye studies of Rous and Gilding (1929). The presence of anaesthesia in both cases makes any direct comparison with the present work difficult. Muscle blood flow measurements would be of value in ischaemic shock, but this is not possible with the present recorders. The partial maintenance of the muscle temperature probably accounts for the failure of the temperature of the mixed venous blood to continue to follow the fluctuations in the liver and brain. In this state of shock the normal order is reversed and the heat of the blood would appear to be mainly derived from muscle.

The low temperature of brain and liver in the later stages of shock is probably related, at least partially, to their poor blood supply. On the other hand, the final brain temperature is usually below that of the liver although the fall in blood flow is less. Furthermore, the results shown in Fig. 9 indicate quite clearly that for about half the survival period the fall in temperature is independent of blood flow changes. While these results are strongly in favour of an intrinsic disturbance of metabolism this cannot be accepted without further argument. The possibility that restoring the circulation to the hind limbs acts like a sudden haemorrhage has been eliminated, but the possibility of the cold tissues of the hind legs acting like a cooling coil in the circulation has not. The majority of the limb vessels must be patent since the tissues stain with fluorescein and their temperature can be raised by perfusion, but in view of the absence of any major temperature recovery the fraction of the blood passing through them is probably small. It is difficult to assess the effect of this on the general body temperature of a conscious animal since hypothermia can only be produced in the homiothermic mammal after inactivation of the compensating mechanisms, *e.g.*, by anaesthesia. The efficiency of these compensating mechanisms in shock is unknown. The main reason for rejecting this possibility as a major factor lies in the difference between the temperature curves for the blood, liver and brain. When hypothermia is produced by cooling the blood stream of the anaesthetised animal the temperature of the liver remains above that of the blood (Ross, 1954). In the present experiments the temperature of the liver and brain fell as soon as the tourniquets were removed and soon fell below that of the blood (Fig. 8). This is particularly clear in the

case of the brain. Since the temperature of the mixed venous blood was frequently higher than that of the organs it had just left, it is unlikely that the fall in the temperature of the liver and brain was due to their being cooled by the circulating blood. So many forces interact in the general response to injury that it is difficult to isolate any mechanism with certainty, but it would seem that at least part of the fall in temperature after injury is due to an intrinsic metabolic disturbance leading to a decrease in energy production.

These temperature changes must also be considered in relation to the effect of limb ischaemia on the stores of chemical energy in the tissues. This has been studied in the rat (Stoner and Threlfall, 1954) where the temperature and blood flow changes (unpublished results) are similar to those described here for the rabbit. At the environmental temperature of the present experiments depletion of the " energy-rich " phosphates of creatine and adenosine was only found in the tissues examined at death. In the undamaged muscle this had only proceeded as far as loss of phosphocreatine, but the adenosine triphosphate level was always reduced in the liver and sometimes also in the brain. These changes are probably due to the severe terminal reduction in the blood supply to the tissues especially the liver, and the beneficial effect of maintaining the circulation to the liver (Frank, Seligman and Fine, 1946 ; Delorme, 1951 ; Wayne, Weir, Barnes, Joyner, Tuttle, Lide and Green, 1954) is probably due to delaying its onset. The absence of change in the stores of " energy-rich " phosphate during most of the shock period, despite the great fall in tissue temperature, must imply a balanced reduction in both the production and utilization of energy.

SUMMARY.

An attempt has been made to decide whether the fall in tissue temperature after injury is due to an intrinsic metabolic disturbance independent of blood flow or is secondary to a decrease in the supply of substrates and oxygen to the tissues following changes in the circulation through them. To this end the temperature of the brain, liver, muscle, mixed venous blood, skin and rectum has been measured and the blood flow through the brain and liver assessed by the method of internal calorimetry in the conscious rabbit during and after a four-hour period of bilateral hind-limb ischaemia. The changes which occurred are described in detail and are compared with those produced by acute haemorrhage and those seen after death. Unsuccessful attempts were made to alter the effects of limb ischaemia by the administration of hexamethonium, dibenamine and terramycin.

Injury appeared to accentuate the temperature-lowering effect of anaesthesia, since the fall in liver and brain temperature during the period of ischaemia was greater than in the controls, although the blood flow changes were the same.

The cause of the fall in tissue temperature after limb ischaemia cannot be absolutely decided since changes in blood flow also occurred. Reasons are given for believing that at least part of the fall is due to direct interference with tissue metabolism. Although the body temperature at death was between 35°–30°, death was not due to hypothermia. When these results are considered in the light of previous chemical findings, it would seem that until nearly the end the blood flow through the brain and liver is adequate for their needs and that there is a balanced depression of both energy production and utilization after injury.

I am indebted to Dr. J. Grayson for help and advice, to Mr. J. C. Wilks for constructing the apparatus, and to Messrs. H. McKinnon and K. Lorrenzen for technical assistance. My thanks are also due to Dr. L. F. Hewitt for supplies of terramycin, and to Messrs. Smith, Kline and French Laboratories for a gift of dibenamine hydrochloride.

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THE INFLUENCE OF THE THYROID GLAND ON SENSITIVITY TO TUBERCULIN IN GUINEA-PIGS.

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THYROXINE increases sensitivity to tuberculin in guinea-pigs (Long and Miles, 1950; Long, Miles and Perry, 1951). However, to produce this increased sensitivity certain conditions have to be fulfilled. Treatment must be continued for at least ten days or preferably longer. In addition, the dose must be exactly judged so that the animals maintain first-rate condition and do not lose weight. A single injection of thyrotrophic hormone, of thyroxine, or of tri-iodothyronine has no significant effect on sensitivity to tuberculin in B.C.G.-infected guinea-pigs, suggesting that the thyroid gland influences sensitivity by some indirect means. The analogy between the influence of thyroid activity on experimental diabetes on the one hand, and on sensitivity to tuberculin on the other (Long, 1954*a*, *b*) leads to the hypothesis that thyroxine influences sensitivity by causing hypertrophy of the islets of Langerhans, the resulting increased insulin output inducing hypersensitivity. In support of this suggestion, injected insulin increases sensitivity to tuberculin in guinea-pigs (Cornforth and Long, 1953).

In the present paper, a factorial experiment is described which explores the inter-relationship of thyroxine, insulin, and insulin deficiency (partial pancreatectomy) in their action on tuberculin sensitivity. Partial pancreatectomy carried out before the tuberculin test is shown to prevent hypersensitivity resulting from prolonged treatment with thyroxine. In addition, hypersensitivity can be restored by injecting insulin.

It is concluded that thyroxine causes increased sensitivity to tuberculin by inducing hyperinsulinism.

METHODS.

The design of the experiment is evident from the first column of Table I, where the seven combinations of treatment with the three factors are listed. Ten albino guinea-pigs of the Hampstead strain, weighing initially about 350 g., were used for each combination; an 8th group of 10 animals served as a control. Animals were injected with B.C.G. vaccine and sensitivity to tuberculin was estimated by the method of Long and Miles (1950). Six months was allowed to lapse between injecting the vaccine and carrying out the tuberculin test; at this time the initial infection with B.C.G. is no longer active and the degree of sensitivity to tuberculin remains remarkably constant. It is probable therefore that effects of treatment are confined to effects upon the established state of sensitivity and that the possibility of effects upon the primary conditioning of cells to allergens during the process of acquiring sensitivity need not be considered.

Sodium thyroxine (0·2 mg./kg.) was injected subcutaneously twice weekly for three weeks. This dose was just sufficient to prevent the animals from gaining weight. A single subcutaneous injection of insulin (1·0 unit/kg.) was given to appropriate groups 1 hr. before the tuberculin test. Partial pancreatectomy or sham operation was carried out under ether

TABLE I.—*Complete Analysis of Variance of Experiment with Combinations of Three Factors.*

Source of variation.	Sum of squares.	Degrees of freedom.	Mean square.	Variance ratio.	Probability.
Between groups	151·4277	7			
A	17·7223	1	17·7223	31·0	<0·001
B	13·9535	1	13·9535	24·4	<0·001
C	71·8910	1	71·8910	125·8	<0·001
A + B	0·3754	1	0·3754	<1	—
A + C	4·6410	1	4·6410	8·1	<0·01
B + C	3·0941	1	3·0941	5·4	<0·05
A + B + C	39·7504	1	39·7504	69·5	<0·001
Between animals	318·6031	72	4·4250	7·7	<0·001
Between doses (linearity)	673·4254	1	673·4254	1178·2	<0·001
Group-dose interaction	3·0777	7	0·4397	<1	—
Residual	41·1531	72	0·5716		
Total	1187·6871	159			

A = Partial pancreatectomy. B = Thyroxine. C = Insulin.

anaesthesia 8–10 days before the tuberculin injections, the intervening time being necessary for wounds to heal before depilation.

In the partial pancreatectomy, from one-half to two-thirds of the pancreas was removed. The head of the pancreas was left intact to minimise damage to the pancreatic ducts, and so to interfere as little as possible with the flow of digestive enzymes into the duodenum. Post-mortem examination showed that in each case an adequate amount of functional pancreas remained.

As in previous work, the diameters of the 24-hr. tuberculin lesions were proportional to the log. of the dose of tuberculin, and the degree of sensitivity could be estimated by the relative positions of the dose-response lines fitted to mean lesion diameters plotted against log. dose.

RESULTS.

A factorial analysis of the experiment (Table I) leads to the following conclusions :

(1) Partial pancreatectomy has a significant effect on sensitivity.
(2) Thyroxine has a significant effect.
(3) Insulin has a significant effect.
(4) Partial pancreatectomy plus thyroxine has no significant effect.
(5) Partial pancreatectomy plus insulin has a significant effect.
(6) Thyroxine plus insulin has a slight but significant effect.
(7) Partial pancreatectomy plus thyroxine plus insulin has a significant effect.

In this type of analysis all the results obtained with different treatments are compared. Thus each of the above conclusions is based on a group of 40 guinea-pigs. Factorial analyses are particularly valuable in demonstrating synergic effects, but they do not provide detailed information. For instance, the first conclusion, that partial pancreatectomy produces a significant effect on tuberculin sensitivity, means that when the responses of the 40 animals subjected to partial pancreatectomy are compared with the 40 sham-operated animals a significant difference is obtained. Further analysis shows however that if the 10 animals in which partial pancreatectomy was carried out, but which were subjected to no other treatment, are compared with the 10 controls, no significant effect is produced. The significant pancreatectomy effect in the factorial analysis has

arisen because the response to thyroxine in sham-operated animals is large compared with that obtained in pancreatectomized animals treated with thyroxine. Similarly, the response to insulin alone is greater than that obtained with insulin in pancreatectomized animals. The deduction then drawn from the analysis as a whole is that partial pancreatectomy prevents the thyroxine effect which is restored by insulin.

Further information about the nature of the relationship of the different factors is obtained by comparison of the direct effects of the individual treatments with the controls. These direct comparisons are shown in Table II. Since they are made with groups of 10 animals only, the advantage of the factorial design, which enables the comparison of effects to be made on groups of 40 animals, is lost.

TABLE II.—*Comparison of Individual Treatment Effects with the Effect in Controls.*

Treatment.	Potency ratio.	Variance ratio.	Probability.
A	1·05	<1	—
B	3·22	59·36	<0·001
C	3·51	90·56	<0·001
A + B	0·92	<1	—
A + C	1·89	21·08	<0·001
B + C	2·65	65·17	<0·001
A + B + C	3·50	67·86	<0·001

A = Partial pancreatectomy. B = Thryoxine. C = Insulin.

Partial pancreatectomy alone has no effect. The significant effect obtained in the factorial analysis is due to the large effects obtained in those groups in which partial pancreatectomy is combined with insulin treatment. Thyroxine alone has a significant effect. Insulin alone has a significant effect comparable in degree with that obtained with thyroxine. Partial pancreatectomy abolishes the thyroxine effect and decreases the insulin effect by approximately one third. Thyroxine and insulin produce significant effects with or without partial pancreatectomy, therefore insulin has restored the effect which was absent in pancreatectomized animals treated with thyroxine.

The evidence that thyroxine influences sensitivity to tuberculin *via* the islets of Langerhans is conclusive. But the plan of the experiment is such that the evidence can be checked and confirmed by additional analyses. The effect of thyroxine on normal animals is 3·35 times greater than that on pancreatectomized animals. Therefore, partial pancreatectomy inhibits the thyroxine effect. Insulin and thyroxine together increase sensitivity in partially pancreatectomized animals 3·62 times as much as thyroxine in partially pancreatectomized animals alone, so that either the presence of insulin has restored the effect of thyroxine or all the response is due to the presence of injected insulin. But insulin plus thyroxine plus partial pancreatectomy is 1·96 times as effective as insulin plus pancreatectomy, showing that thyroxine has been effective. This is supported by the slight but significant interaction between thyroxine and insulin in the full factorial analysis (Table I). This slight effect is to be expected, for if thyroxine causes hypertrophy of the islets of Langerhans associated with increased sensitivity to tuberculin, then the natural production of insulin will be greater in partially pancreatectomized animals treated with thyroxine than in partially pancreatec-

tomized animals not treated with thyroxine. This slight effect was only demonstrable because previous experience enabled us to judge the dose of insulin and thyroxine so that neither effect was maximal and yet both fell on the steepest part of the dose-response curve.

DISCUSSION.

The analogy between susceptibility to experimentally induced diabetes and sensitivity to tuberculin is strong. Diabetogenic agents tend to decrease, and anti-diabetogenic agents to increase, sensitivity to tuberculin (Long, 1954*a*, *b*). The relationship of thyroid activity to these effects is important. On the one hand, propylthiouracil prevents desensitization by cortisone (Long *et al.*, 1951), whereas thyroxine increases sensitivity (Long and Miles, 1950). On the other hand, thiouracil reduces the incidence of diabetes in rats subjected to partial pancreatectomy, and thyroid administration at first accelerates the appearance of diabetes and then cures it by inducing hyperplasia and hypertrophy of the islets (Houssay, Foglia and Martinez, 1946). Therefore, hypo- or hyper-thyroidism oppose the production of diabetes or of desensitization.

Houssay (1950) attributes the beneficial effect of hypothyroidism to an increase in the free -SH groups of the tissue, and that of hyperthyroidism to an increase in islet tissue associated with hyperinsulinism (Houssay *et al.*, 1946). Continuous thyroxine treatment causes hypertrophy of the islets in guinea-pigs. The findings reported here strengthen even further the analogy between experimental diabetes and sensitivity to tuberculin, and are consistent with the Houssay hypothesis. It is probable that -SH metabolism is intimately concerned with sensitivity to tuberculin (see Long, 1954*b*).

SUMMARY.

Using the response to intradermal tuberculin as a measure of allergic hypersensitivity in albino guinea-pigs sensitized with B.C.G., it was found that :

(1) thyroxine increases sensitivity ;
(2) insulin increases sensitivity ;
(3) partial pancreatectomy has no effect on sensitivity but prevents the action of thyroxine in increasing sensitivity ;
(4) partial pancreatectomy does not prevent the increase in sensitivity that follows injection of insulin.

It is concluded that thyroxine increases sensitivity to tuberculin in guinea-pigs by increasing the amount of islet tissue and so inducing hyperinsulinism.

We are indebted to Dr. Knud Tolderlund of the State Serum Institute, Copenhagen for the B.C.G., to Miss M. V. Mussett, B.Sc., for the statistical analyses and for advice on their interpretation.

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ACTIVATION OF A HISTAMINE-RELEASING AGENT (ANAPHYLATOXIN) IN NORMAL RAT PLASMA.*

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IN previous reports from this laboratory (Rocha e Silva, Aronson and Bier, 1951 ; Rocha e Silva, 1952 ; Rocha e Silva and Aronson, 1952) it was shown that anaphylatoxin or serotoxin, prepared according to the method described by Bordet (1913), is able to release considerable quantities of histamine when perfused through the isolated guinea-pig lung. This histamine-releasing activity of normal rat plasma treated with agar can also be demonstrated by simply adding the " activated plasma " to the chamber containing a piece of guinea-pig ileum, in a Dale's apparatus. Rat plasma incubated for a few minutes with agar acquires the property of eliciting a strong contraction of the isolated guinea-pig ileum. That this contraction is the result of the release of histamine from the gut itself could be demonstrated since : (*a*) this stimulating effect can be completely blocked by anti-histaminics and not by atropine ; (*b*) de-sensitization (tachyphylaxis) follows a number of additions of the same dose of the " activated " plasma or serum ; (*c*) this tachyphylaxis is most probably a result of exhaustion of the store of bound histamine since the gut's sensitivity to histamine remains unimpaired. The present work is a study of the mechanism by which this histamine-releasing agent becomes activated in rat plasma or serum under varying experimental conditions.

MATERIALS AND METHODS.

Preparation of anaphylatoxin (activated plasma).

Blood was collected from the lightly (ether) anaesthetized rat by heart puncture, into a syringe containing 1 mg. heparin/10 ml. blood. Several animals were bled successively and the plasma of the pooled samples collected by centrifugation. The clear plasma was mixed at once with the indicated amounts of polysaccharides and incubated for 10–30 min. at 37°, unless otherwise stated. Usually 0·05–0·10 ml. of a diluted (1/2) and fully activated plasma produced a maximal contraction of the gut. In many experiments plasma was activated by dilution to 1/10 or less with distilled water and incubated for 10 min. at 37°. Activation could also be obtained by dialysis of plasma against distilled water for 2 hr. at room temp. Most of the experiments were done with fresh plasma or plasma kept for 1 or 2 days in the frozen state.

Assay on the guinea-pig ileum.

The method of detecting activation of anaphylatoxin by its effect on the isolated guinea-pig ileum has been employed as a routine assay. The chamber containing the piece of gut had the capacity of 4·0 ml., and the conditions of the assay are the same as those described in previous papers from this laboratory. In most of the experiments a frontal writing lever

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of the type described by Schild (1944) has been used. Tyrode solution at pH 7·4 was used as perfusing fluid. The standard histamine solution had a concentration of 0·05 mg./100 ml. calculated as the base. Additions to the chamber were made after a 1 min. interval after washing out the gut with fresh Tyrode solution. Pieces of ileum from a freshly killed guinea-pig were always used, since it was found that the responsiveness of the gut to activated plasma decreased fairly rapidly when the gut was kept in Tyrode solution at room temp. or in the refrigerator for more than a few hours, probably as a result of diffusion or destruction of the bound histamine.

Sulphation of the polysaccharides.

Agar, dextran and starch were sulphated as described by Karrer, Koenig and Usteri (1943), using chlorosulphonic acid in pyridine below 0° in a dry atmosphere. After slowly adding the dried polysaccharide the mixture was heated for several hours at 60–80°, and the sulphated polysaccharide extracted with cold water and precipitated with ethanol. The precipitated material was collected by filtration, washed with ethanol, suspended in water and re-dissolved after alkalinization with NaOH. The solution was dialysed for 48 hr. against running tap water and re-precipitated 1 or 2 times in the same manner, using ethanol. The final precipitate was washed with acetone and dried *in vacuo*. The brownish or yellowish loose powder thus obtained gave a strong metachromatic colour with toluidine blue.

Fractionation of the hydrolysates from starch.

Acid hydrolysis of a commercial potato starch (defatted by continuous extraction with ethyl ether) was obtained by submitting 40 g. of the raw material, suspended in 800 ml. 0·1 N-HCl, to 10 min. heating at 100°. The solution was neutralized with NaOH and the different fractions obtained by fractional precipitation with increasing amounts of ethanol. Each fraction was collected by centrifugation and dried over alcohol and acetone. The coefficient " Total glucose/reducing glucose " for each fraction was obtained by estimating the total hydrolysable glucose and the terminal " free " reducing glucose by the iodine titration method according to Cajori (1922).

Decalcification of rat plasma.

Rat plasma was decalcified by passing it through a column of the cationic ion-exchange resin Dowex 50, which had been previously washed with HCl, NaOH, concentrated NaCl and saline. The plasma so treated no longer gave any reaction with sodium oxalate.

Effect of salts on the activation of rat plasma by agar.

An amount of each salt calculated to give the desired concentration in the final mixture was dissolved in 0·5 ml. saline and added to 0·5 ml. of the agar solution (0·01 per cent) in saline and 0·5 ml. rat plasma. The mixtures were incubated for 10 min. at 37° and when not assayed immediately, were kept in crushed ice until tested. All incubations were at pH 7–7·4. The anaphylatoxin assay was performed by adding 0·05 or 0·10 ml. samples to the perfusion bath ; with such amounts, only rarely did the controls containing salt and plasma alone have any effect on the gut. The mixtures containing the highest salt concentrations (*i.e.*, affording the greatest protection from activation) were tested first, until the concentration affording threshold activation had been reached ; samples containing agar and different levels of sodium chloride as inhibitor were tested as controls, at the end of the experiment.

Agar.—The stock solution of agar (Difco), was routinely made by suspending the weighed material in saline and heating to the boiling point for a few minutes ; usually a 0·25 per cent solution of agar thus made was stored in the ice box for a few weeks. Before using the solution it was again gently heated to facilitate pipetting. The *soluble starch* (Mallinkrodt Chemical Works) had, according to our method of determination (alkaline iodine titration), an average total of 35 glucose units per reducing group. Previous to this titration, the material had been incubated for 16 hr. with yeast to eliminate glucose and maltose.

Two kinds of *dextran* were employed : " crude dextran " was prepared from a culture of *Leuconostoc mesenteroides* by deproteinization and alcohol precipitation and kindly supplied by Dr. H. Moussatché, of the Oswaldo Cruz Institute in Rio de Janeiro ; " partially hydrolysed dextran " (batch TDR 221) was kindly supplied by Pharmacia from Uppsala, Sweden.

Alginic acid and its sulphated derivative (*Paritol*) were kindly supplied by the Wyeth Institute of Applied Biochemistry, Philadelphia, U.S.A.

Three different kinds of bacterial polysaccharides from *Bacillus anthracis* and from the somatic antigen of *Salmonella typhi* and *Salm. paratyphi* were kindly provided by Dr. Anne Marie Staub, from the Institut Pasteur, Paris.

Inulin.—A clinical brand of the type employed for renal clearance tests was used.

Glycogen.—Dog's liver glycogen was prepared in our laboratory by precipitation with alcohol from a trichloracetic filtrate of the macerated organ.

Kollidon (poly-vinyl-pyrrolidone).—Three preparations of different molecular weights of the order of 500,000 (K_1), 47,000 (K_2) and 11,000 (K_3) were assayed. These materials were kindly supplied by Prof. H. Weese, Bayer A. G., Elberfeld.

Peptone.—Bacto peptone (Difco) was used.

Heparin.—A commercial preparation (Vitrum) containing 50 mg./ml. was generously supplied by Apo Vitrum Comercial of S. Paulo, Brazil.

Ovo-mucoid.—A preparation obtained in our laboratory according to the method described by Meyer (1945) was used.

Soy-bean trypsin inhibitor.—A 5-times recrystallized preparation was kindly supplied by Dr. M. Kunitz of the Rockefeller Institute for Medical Research, Princeton, N.J.

Specific precipitate from rabbit serum immunized to egg albumin.—The washed serum-free suspension was kindly supplied by Dr. Maria Siqueira from the Department of Immunology of the Instituto Biologico, S. Paulo, Brazil.

Sulphated polysaccharides.—The sulphated products of cellulose glycol ether, chondroitin sulphuric acid and of pectin, were kindly supplied by Prof. P. Karrer of Zürich Polytechnic Institute, Switzerland.

Salts.—The inorganic and organic salts used in the salt inhibition experiments were of analytical grade, and were dried to constant weight before use, unless a hydrated form of known formula was used.

RESULTS.

Effect of Rat Anaphylatoxin on the Guinea-pig Ileum.

Heparinized normal rat plasma added to the bath containing a piece of guinea-pig ileum elicits a small contraction when added in doses of 0·3–0·5 ml. Usually 0·2 ml. or less produces only a slight change of tonus accompanied by rhythmic contractions. When the same plasma is incubated for a few min. with agar, it will elicit a powerful tonic contraction, similar to that produced by histamine, as shown in Fig. 1. Successive additions of the same dose of " activated plasma " (anaphylatoxin) will produce a lesser and lesser effect and finally the muscle is de-sensitized or tachyphylactic to it. That this tachyphylaxis is due to a de-sensitization effect and not to a destruction of the spasmogenic agent by further contact with the plasma, could be demonstrated by changing the piece of ileum. The new piece will react with the same dose of anaphylatoxin, again with a strong contraction followed by de-sensitization. Since anaphylatoxin prepared by incubation of normal rat plasma with agar is very potent as a histamine releaser when perfused through the isolated lung of the guinea-pig, it was assumed that this effect on the guinea-pig gut is also due to a release of histamine. Atropine in concentrations which completely block acetylcholine had no effect on the contraction produced by anaphylatoxin, and anti-histaminics (Antistin or Neo-antergan) in concentrations that had no effect on the spasmogenic action of acetylcholine, completely blocked the contraction produced by threshold doses of anaphylatoxin, as shown in Fig. 2. In a few cases, however, a slow contraction of the gut could be obtained even in the presence of Neo-antergan. As de-sensitization proceeds, the latent period, which is very short after a first addition of anaphylatoxin, becomes longer and longer, until the muscle becomes completely unresponsive.

The constancy of the stimulating effect of rat anaphylatoxin on the guinea-pig ileum contrasts with the irregular and inconstant results obtained with

anaphylatoxins prepared from guinea-pig serum (Dale and Kellaway, 1922), or from the serum of horse, rabbit or pig (Adant, 1950). In a long series of experiments only occasionally did the agar-activated plasma fail to produce a strong contraction. However, usually this failure was due to the unresponsiveness

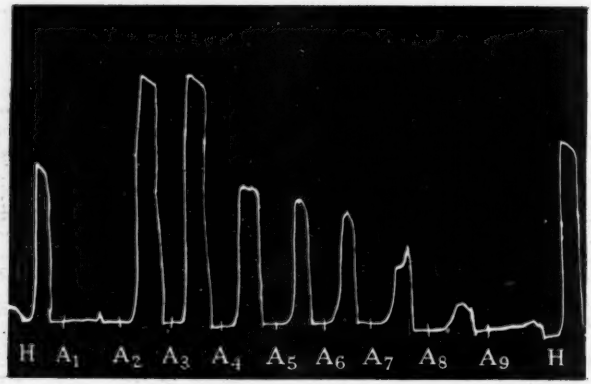

FIG. 1.—A_1 means a sample (0·15 ml.) taken from the mixture of rat plasma with 0·1 mg. of agar per ml., after incubation at 37° for 20 sec. A_2, A_3 etc. indicate additions of equal samples taken every 3 or 4 min. from the same incubating mixture. Note progressive de-sensitization of the gut toward anaphylatoxin. H, 0·05 μg. histamine (base).

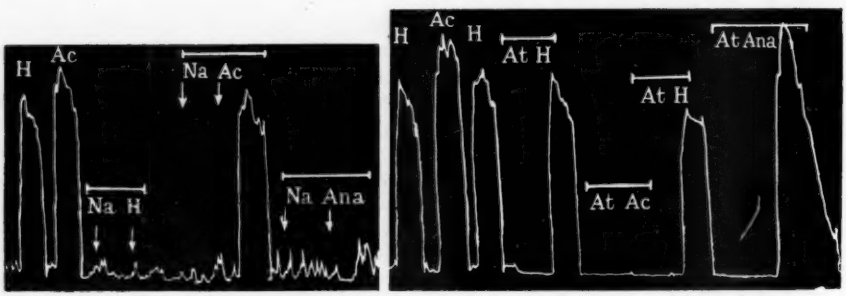

FIG. 2.—Effect of Neo-antergan and atropine on the action of anaphylatoxin on the guinea-pig ileum. H, 0·1 μg. histamine (base). Ac, 0·08 μg. acetylcholine. NA, 2·0 μg. Neo-antergan (anthisan maleate). At, 2·0 μg. atropine. Ana, 0·25 ml. rat plasma activated by incubation with 0·1 mg./ml. agar.

of the gut rather than to the absence of activity in the plasma, since by taking the piece of ileum from a new guinea-pig, the response to the activated material was usually obtained.

In spite of the de-sensitization effect, semi-quantitative results could be obtained in the same piece of gut in many cases. For instance, in the experiment presented in Fig. 3, the same rat plasma was incubated with increasing doses of starch. As little as 100 μg. produced a threshold effect, although full activity was

only obtained with 10 mg. starch. Under similar conditions agar was about 10 times more potent, affording full activation with 1 mg./ml.; but doses as small as 5 to 10 μg. agar produced a detectable effect when incubated with 1 ml. rat plasma.

Many different substances were tested for capacity to activate rat plasma (Table I). As an approximate indication of their relative activating potencies their effects are compared to those given by adding an equivalent amount of agar-activated plasma to the same gut.

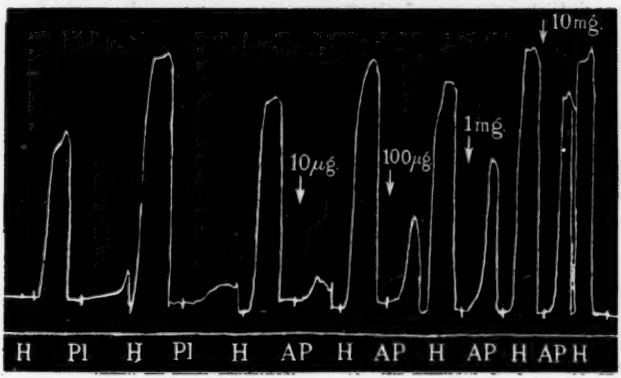

FIG. 3.—Effect of the dose of activator (potato starch) on the degree of activation of rat plasma. H, 0·05 μg. histamine (base). Pl, 0·2 ml. rat plasma diluted 1/2 with saline. AP, 0·1 ml. rat plasma incubated for 10 min. at 37° with varying amounts of starch. The amounts shown are wt. starch/ml. plasma.

The ability of starch to produce activation of rat plasma depends on the degree of polymerization. Non-hydrolysed starch was assayed in comparison with 5 different fractions of an acid hydrolysate of starch obtained by fractional precipitation with increasing concentrations of ethanol (Fig. 4). It is clear that the fractions containing the longest chains of split products (precipitated at lower concentrations of ethanol) are the most effective in producing activation. To have an idea of the relative chain lengths of these fractions, their reducing powers were estimated by the alkaline iodine titration method as described, and the ratio "total glucose/reducing glucose" expressed as shown in Table II. The colours produced by iodine with the different fractions are also stated.

Effect of Temperature on the Activation of Plasma by Polysaccharides.

As shown before (Rocha e Silva and Aronson, 1952) heating normal plasma for 30 min. at 56°–60° completely destroys its capacity for activation by polysaccharides, although the already active anaphylatoxin is somewhat more resistant to heat, since 1 hr. at 69°–70° caused only a partial inactivation of pre-formed anaphylatoxin. Concerning the effect of temperature on the activating process, the results presented below definitely indicate that a chemical or enzymic reaction is going on as a consequence of the interactions of the polysaccharide with the plasma proteins. As shown in Fig. 5, at 0°–2° incubation with 1 mg. agar/ml.

TABLE I.—*Compounds Tested as Activators of Rat Plasma Anaphylatoxin.*

	Dose (mg./ml. plasma).	Effect.
Agar-agar	0·05	+++
Starch (soluble)	0·1	++
„ (potato)	0·1	++
Dextran (crude)	0·1	+++
„ (partially hydrolysed)	1·0	+
Inulin	1·0	+
Specific precipitate from rabbit anti-ovo-albumin serum and ovo-albumin	1·0	++
Sodium alginate (Manucol)	2·0	+
Polyvinyl pyrrolidone (Kollidon)	2·0	+
Glycogen (dog's liver)	5·0	+
Polysaccharides from :		
(*a*) Somatic antigen of *Salm. typhi*	20·0	++
(*b*) „ „ *Salm. paratyphi*	20·0	+
(*c*) *B. anthracis*	20·0	++
Peptone	20·0	0
Heparin	25·0	0
Ovomucoid	20·0	0
Kaolin	20·0	0
Silica-gel	5·0	0
Sulphated products from :		
(*a*) Agar-agar	20·0	0
(*b*) Dextran	20·0	0
(*c*) Starch	20·0	0
(*d*) Alginic acid	20·0	0
(*e*) Chondroitin sulphuric acid	20·0	0
(*d*) Pectin	20·0	0

For each test, weighed amounts of each compound were dissolved in 0·5 ml. saline, mixed with 0·5 ml. plasma and incubated at 37° for 30 min.

0, no activation ; + weak activation ; ++, moderate activation ; +++, maximum activation.

TABLE II.—*Properties of Potato Starch Hydrolysates.*

Fraction.	Vol. of alcohol needed for precipitation (per unit vol. of hydrolysate).	Colour with iodine.	Total glucose / Reducing glucose.
I	1	Blue	37·0
II	2	Violet	33·4
III	3·5	Red	15·9
IV	5·5	Brown	11·9
V	7·5	None	9·1

plasma failed to produce any detectable activation even after 4 hr. At 10° approximately 60 min. and at 20° 20–30 min. were required to produce a threshold activation effect. At 27° activation was somewhat slower than at 37°, when it was complete in 3–5 min. At 47° activation of this plasma sample by agar was almost instantaneous, since maximal activity was observed after only 1 min. incubation. These results were obtained with one sample of pooled plasma, variations in the time-temperature relationship having to be expected between one plasma and another. The high temperature coefficient apparent for this process suggests that a chemical reaction probably of enzymic nature is involved.

As mentioned before, activation of rat plasma could also be obtained by dilution to 1/10 or less with distilled water, without addition of any activator. In Table III the influence of temperature on activation by dilution is shown. In this

case the influence of temperature is much smaller, although still perceptible at 0°–2°. However, at a dilution of 1/3 the influence of temperature on activation is more evident. Thus, at the lowest level, no full activation was observed even after 5 hr. incubation; at 18° the process is still very slow, complete activation requiring about 1 hr. These time intervals should be compared with that at 37°, where full activation was obtained in 7–10 min.

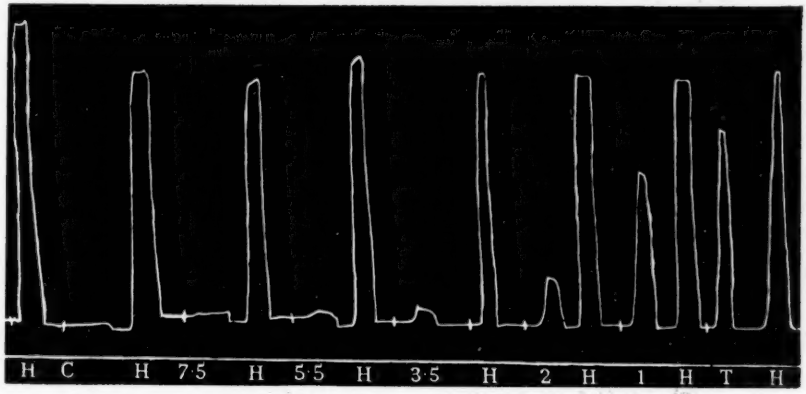

FIG. 4.—Effect of chain length on the activation of rat plasma by various fractions obtained from a potato starch hydrolysate. C, 0·1 ml. rat plasma diluted 1/2 with saline. 7·5 to 1–0·05 ml. rat plasma incubated for 30 min. at 37° with fractions of the hydrolysate precipitated by 7·5, 5·5, 3·5, 2·0 and 1·0 volumes of alcohol respectively. In each case the plasma was incubated with 10 mg./ml. of the activator dissolved in 1 ml. saline. T, incubated with total non-hydrolysed starch. H, 0·05 μg. histamine (base).

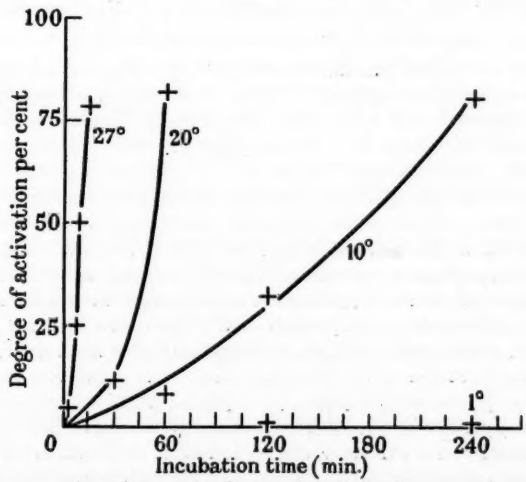

FIG. 5.—Effect of temperature on the rate of activation of rat plasma by agar. Ordinates represent the relation between the activation of plasma incubated with 1 mg./ml. agar at different temperatures and the maximal activation obtained with agar.

TABLE III.—*Influence of Temperature on the Spontaneous Activation of Rat Plasma after Dilution with Distilled Water.*

Incubation (min.).	Dilution 1/10. Temperature				Dilution 1/3. Temperature		
	1°	18°	27°	37°	1°	18°	27°
3	0	+	++	+++	0	0	++
5	0	+++	+++	+++	0	0	+++
10	0	+++	+++	+++	0	0	+++
15	0	+++	+++	+++	0	+	+++
35	+++	+++	+++	+++	0	+	+++
60	+++	+++	+++	+++	0	+	+++
300	+++	+++	+++	+++	+	+++	+++

0, no activation ; +, threshold activation ; ++, moderate activation ; +++, maximal activation.

It thus appears that activation by dilution is, like activation by agar, not an instantaneous process, but a chemical, probably enzymic, effect, with a high activation energy. In order to explain the quicker effect of the 1/10 dilution compared with that at lesser dilutions, it would be reasonable to assume that the activator is liberated in greater amounts at this dilution, than at 1/3. It seems unnecessary to postulate the existence of two different thermolabile activators, one for agar and another for activation by dilution, since in both cases heating the plasma for 56°–60° for 30 min. completely destroys its capacity to be activated by either agent. However, the process involved in activation by agar might depend on another step, *i.e.*, the action of agar itself releasing the activator, which will in turn act enzymically on the precursor of anaphylatoxin, setting anaphylatoxin free. The shape of the curves in Fig. 5, with the concavity turned upwards, could not be reproduced with material diluted 1/3. The effect of temperature followed in this case a more straightforward pattern, the tendency of the curve being to bend downwards.

Activation of Anaphylatoxin by Dialysis.

The mechanism by which plasma is activated by dilution was studied further, by submitting it to dialysis against different ionic strength solutions. Dialysis for 2 hr. against distilled water at room temperature caused definite activation. No such effect was obtained by dialysis against saline (ionic strength 0·15). Maximal activation was obtained after 12 hr. dialysis against distilled water at 8°. Under such conditions dialysis against saline (ionic strength 0·15) produced only a small activation which could be greatly increased by diluting an aliquot of the dialysed plasma with distilled water to 1/10. No such increase could be obtained by diluting plasma which had been dialysed against distilled water. It was further observed that no activation could be produced by diluting plasma with saline (1/10), followed by incubation at 37° for up to 30 min. ; under similar conditions dilution with 3 vol. distilled water produced a high degree of activation within 30 min.

Effect of Ions on the Activation of Anaphylatoxin by Agar.

The above experiments strongly suggested that activation of anaphylatoxin depends on the ionic strength of the medium. This idea was corroborated by the finding that in a medium of approximately twice the ionic strength of saline (0·30–0·35) activation of rat plasma by agar was always prevented. Subsequent

dilution to normal saline level (ionic strength 0·15) allowed activation to proceed normally. A study of a series of cations and anions which proved to be effective as inhibitors of the process was therefore made. As shown in Table IV the anions

TABLE IV.—*Effect of Anions on the Activation of Anaphylatoxin by Agar.*

Anion.	Average "threshold" concentration.	Average "threshold" ionic strength.
SO_4^{++}	0·12-N	0·18
Cl^+	0·12-N	0·12
I^+	0·06-N	0·06
NO_3^+	0·06-N	0·06
SCN^+	<0·06-N	<0·06
Salicylate$^+$	<0·06-N	<0·06
Oxalate^{++}	0·04-N	0·06
Citrate^{+++}	<0·04-N	<0·12
	>0·02-N	>0·06

Each result is the average of two or more determinations of the salt concentrations just sufficient to allow for a beginning activation of rat plasma by agar (0·1 mg./ml.). All salts were assayed as sodium or potassium compounds.

could be arranged in the following order: citrate $\geqslant$ oxalate $>$ salicylate $>$ thiocyanate $>$ nitrate $=$ iodide $>$ chloride $\geqslant$ sulphate, when the concentrations allowing threshold activation are presented in normalities. However, if the ionic strengths of these solutions are compared, the differences between citrate, oxalate and chloride are much less pronounced and the effect of sulphate becomes significantly smaller than that of chloride. Still the differences between salicylate, iodide, thiocyanate and chloride will not be reduced so that in the case of the monovalent ions, the size of the charged particles appears to constitute a factor in the inhibition of the activation process by electrolytes. In separate experiments more direct comparisons between different anions were made. For instance, thiocyanate was always more potent than iodide and nitrate, although nitrate and iodide did not show substantial differences. These additional data permitted a better localization of the different ions in the series. As regards cations, comparisons were made with only a few, the results, as well as those using urea and glucose being given in Table V. There was no significant difference between the monovalent cations (Na^+, K^+ and Li^+). In the divalent series, however, Ca^{++} was definitely stronger than Mg^{++} and about as active as Sr^{++}. Other cations such as Ba^{++}, Zn^{++} or Al^{+++}, could not be properly assayed because of the effects on the gut of such doses as might prevent activation.

Action of Sulphated Polysaccharides on Rat Plasma.

In view of the increasing importance of the sulphated polysaccharides as heparin substitutes, the action on rat plasma of starch, dextran and agar sulphates was studied. As it can be seen from Table I, these three most active polysaccharides entirely lost their anaphylatoxin-activating capacity after being sulphated. Even doses twenty times higher than those of the non-sulphated products giving maximum contraction failed to produce any effect. Heparin, also a sulphated derivative of a polysaccharide, was equally ineffective when assayed in doses of 5, 10 and 25 mg./ml. plasma. However, when assayed in connection with agar, heparin showed an inhibitory action, by completely preventing agar activation of plasma when present in the plasma previous to the addition of the agar, at a final concentration of 15 mg. heparin per ml. At the 10 mg./ml. level, this inhi-

TABLE V.—*Effect of Cations and Neutral Compounds on the Activation of Rat Plasma by Agar.*

Cation.	Average "threshold" concentration.	Average "threshold" ionic strength.
Na^+	0·12-N	0·12
K^+	0·12-N	0·12
Li^+	0·12-N	0·12
Mg^{++}	0·06-N	0·09
Ca^{++}	0·02-N	0·03
Sr^{++}	0·02-N	0·03
Glucose	>0·50-M	
Urea	0·25-M	

The determinations were carried out as described for Table IV. All salts were assayed as chlorides.

bition was only partial, while at the 5 mg./ml. level, activation was indistinguishable from that of the controls containing the usual concentration, enough to prevent coagulation.

DISCUSSION.

The striking stimulating effect of rat anaphylatoxin on the isolated ileum of the guinea-pig afforded a simple and reliable technique to study the conditions under which activation of the histamine-releasing principle takes place, under varying experimental conditions.

Although a slight activation of normal rat plasma was observed even when the plasma was left for several hours at 37° or at room temperature, the process could greatly be accelerated by adding minute amounts of polysaccharides, or by diluting plasma or dialysing it against distilled water. Amounts of agar of the order of 0·02 mg./ml. plasma produced a perceptible effect, and dilution with an equal volume of distilled water, followed by a 6 minutes' incubation at 37°, also conferred definite histamine-releasing activity. Although no conclusive evidence is offered of an identity of the mechanisms of production of the polysaccharide-activated anaphylatoxin and the histamine-releasing principle appearing after dilution or dialysis, several facts strongly point in this direction, both processes being highly dependent upon temperature, inhibited at higher ionic strengths and by the previous heating of plasma to 56°.

In an effort to shed some light upon the nature of the interaction between the activating polysaccharide and the plasma proteins, some structural differences between active and non-active polysaccharides were analysed. The chain lengths of the different fractions of hydrolysed starch greatly affect their capacities to activate anaphylatoxin, the shorter the chain length, the smaller being this effect. Possibly the observations of Ingelman (1949) and others regarding the direct relationship between chain length and toxicity of dextran could be explained on such a basis, since dextran is an efficient activator of rat plasma anaphylatoxin. The presence of strongly charged negative substituents on the polysaccharide chain could be shown to be another factor capable of reducing or even abolishing its anaphylatoxin-activating capacity. Thus, products obtained by sulphation of agar, dextran or starch, and the carboxyl-containing alginates had no or very slight activity. Moreover, it was noted that in stronger concentrations the sulphated polysaccharides could antagonize activation of rat plasma by agar. This effect, also observed with heparin, might be explained as being due to a block resulting from a salt-like combination between the negative sulphate groups of the

polysaccharides and some positive grouping of the plasma proteins, which are involved in the activating process either as precursors or as enzymic activators of anaphylatoxin. As shown by Jaques (1943), heparin is capable of reacting at physiological pH with several plasma proteins.

The activation of rat plasma by agar is greatly dependent on the ionic strength of the medium. In the systematic study of the threshold concentrations of several salts which permitted activation by agar, it was observed, however, that besides ionic strength, more specific effects could be observed. Thus anions could be arranged in this order of decreasing effectiveness : citrate $\geqslant$ oxalate $>$ salicylate $>$ SCN $>$ I $=$ NO_3 $>$ Cl $>$ SO_4, while in the cationic series the sequence Ca $>$ Mg $>$ 2Na was found. Except for citrate and oxalate, the position of the anions in this series resembles closely that of anions of the lyotropic series. A similar sequence has been shown by Marrack (1934) to hold for the relative inhibiting power of anions on the formation of the specific precipitate.

Gordon and Thompson (1933) have shown a similar effect of anions on the inhibition of complement in the haemolysis of sensitized ox red blood cells by guinea-pig serum. Edsall and Lever (1951) also demonstrated such an anionic sequence in the inhibition of the clotting of fibrinogen by thrombin. That Ca^{++} ions are more effective than Na^{+} in the prevention of this process has also been shown to hold true by Edsall, although it is not yet clear whether this action is truly an inhibition of the proteolytic action of thrombin upon fibrinogen or merely an inhibition of the polymerization of " activated " fibrinogen into fibrin.

In a previous publication (Rocha e Silva and Aronson, 1952) it had been assumed that sodium citrate inhibited the activation of anaphylatoxin either by removing Ca^{++} ions from the medium or by preventing the disintegration of platelets, and it was thought possible that platelet disintegration could furnish the activator or a co-factor for activation of anaphylatoxin. More recent results have shown, however, that the presence of free Ca^{++} ions is not needed for anaphylatoxin activation, since decalcified plasma obtained by passage through a Dowex 50 resin column can still be normally activated by agar. As far as the second hypothesis is concerned it would appear that sodium citrate, as well as the other anionic inhibitors tested would rather act on plasma proteins blocking the precursor or activator of anaphylatoxin. The observation that salt inhibition proved equally effective either when tested upon plasma or upon serum points towards this view. However, the fact that citrate and oxalate may not act solely by means of ionic strength effects cannot be excluded.

McIntire, Roth and Richards (1949) showed that relatively high doses of heparin as well as citrate, oxalate, Ca^{++} and Mg^{++} were able to inhibit or reduce the *in vitro* shift of histamine from blood cells to plasma of sensitized rabbits when antigen was added. Comparing the doses required in McIntire's experiments with those needed to inhibit anaphylatoxin, a rather close similarity can be seen in nearly all cases. These facts, as well as the incapacity of soy-bean trypsin inhibitor to affect either the activation of anaphylatoxin by agar or the anaphylactic release of histamine in rabbit blood, strongly suggest that a similar mechanism is involved in both reactions.

As regards the relationship between the facts presented in this paper and the intimate mechanism of anaphylaxis, a general discussion has been presented before (Rocha e Silva, 1953). It is clear that anaphylatoxin can be activated under such conditions as those existing in anaphylactic shock. The specific

precipitate obtained by the interaction of ovo-albumin, for instance, and the serum of rabbits immunized to it, can replace the polysaccharides in the process of activation of anaphylatoxin. This fact, known for a long time, has been confirmed in our laboratory using the isolated ileum of the guinea-pig and rat plasma, to show activation of anaphylatoxin by the specific precipitate. However, recent experiments by Schachter (1953) showing that sensitized rabbit organs perfused with antigen in Locke solution in the absence of blood are able to release histamine, might indicate that some direct interaction of the antigen with the cells is a sufficient stimulus for the release of histamine. However, the impossibility of removing all traces of blood from a perfusion system, as well as the probable presence of anaphylatoxin in the interstitial fluid (lymph), might be factors in histamine release under the conditions of Schachter's experiments.

SUMMARY.

Rat plasma, incubated with agar, starch, dextran and several other polysaccharides, acquires the capacity to release histamine from the guinea-pig ileum, as shown by the powerful contraction elicited *in vitro* followed by desensitization. Dilution or dialysis of the plasma against distilled water followed by a brief incubation at 37° has a similar effect.

Activation both by agar and by dilution are strongly dependent on temperature, the former having a higher temperature coefficient than the latter.

The length of the polysaccharide chains constitutes an important factor, the products having a shorter chain being less efficient activators than those with longer chains.

Sulphation of the polysaccharides suppresses their activating capacity. Relatively small increases in ionic strength of plasma inhibit its activation by agar. A systematic study of the relative inhibitory powers of several anions showed that their relative efficiencies paralleled their positions in the lyotropic series. Among the cations studied, Ca^{++} proved to be much more effective as inhibitor than Mg^{++}, Na^{+}, K^{+}, or Li^{+}.

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